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THE CANCER DEATH RATE IN THE COLOURED POPULATION OF ANTIGUA, WEST INDIES, OVER THE LAST SEVENTY YEARS

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ANTIGUA, an island of 108 square miles, is situated in latitude 17° north and longitude 62° west of Greenwich, in the Leeward Islands, which lie between Puerto Rico to the West and the Windward Islands and Barbados to the South-east. Throughout its history its inhabitants have been engaged in agriculture, and the single town contains only 12,000 inhabitants. The total population, excluding that of Barbuda, a dependency 30 miles to the north, with which this paper is not concerned, is at present 55,000; its population in 1887 the date with which this survey begins was about 35,000. The great majority of Antiguans are negroes and people of mixed descent. During the period under review there were never more than 4 per cent of the population who were white, and since 1900 the proportion has dropped to less than 1 per cent.

Data for the Survey

Antigua has been fortunate in that fairly reliable censuses have been taken at intervals since 1841, namely, in 1861, 1871, 1881, 1891, 1901 (totals of the two sexes only), 1911, 1921 and 1946. The censuses with which we are at present concerned, those for 1891, 1911, 1921 and 1946, appear to have been well carried out, and probably not many persons were missed. One likelihood of error, however, which is always present in such censuses in undeveloped countries concerns the declared ages of persons when interviewed by the census officers. Births and deaths have been recorded since 1856, and, as will be discussed in another paper, records became complete fairly quickly. The Marriages, Births and Deaths Act of 1870 requires all deaths to be registered, and no body may be buried without a burial order; the granting of which is dependent on the furnishing of either a doctor's or a coroner's certificate. The evidence of the death registers is that Antigua has probably been much more fortunate than many West Indian territories in having had a reasonably good supply of physicians throughout the period under consideration. My perusal of the death registers and the collection of my data go back to the beginning of deaths registration, but I have not included statistics about malignant neoplasms before 1887, because vague terms such as senility, cachexia, old age and many others of a similar nature were in common use in the first few decades, and it was evident that many cancers were hidden under these unsatisfactory terms.

This survey is therefore concerned with all the cancer deaths registered as having occurred in coloured people in Antigua in the years 1887 to 1957 inclusive; they number 1410 in all. No deaths among the white population are included. In the early years the term "cancer" or "sarcoma" without any indication as to its position in the body was common; this accounts for the large number of deaths under No. 199 in Table III, but there was a noteworthy drop in this manner of labelling cancer deaths shortly after the turn of the century, and it has not been commonly met with for nearly forty years, though a tendency to diagnose the cancer from its metastatic rather than from its primary site persisted until after the recent war.

All deaths under consideration come under the headings of the International Statistical Numbers 141 to 204 in the International List of Causes of Death.

As regards the completeness of notifications and the accuracy of the death returns, these vary from place to place and from country to country, so that it is very likely that international comparisons which I have drawn in this paper in many instances may be much less comparable than perhaps I may consider likely in any particular instance. This is a matter to be borne in mind particularly by readers in Britain and in the United States. On the other hand, as Wilcocks (1932) has truly said: "It should not be overlooked that the observers of long ago were no more lacking in clinical acumen than (present day) men who have so many more advantages."

In interpreting the tables in this paper it is necessary to realise that over the last hundred years, especially during years of economic depression, young adults have emigrated, at times in sizeable numbers, though until the last thirty years no record has been kept of these population movements. During the construction of the Panama Canal many young men left the Colony for work on the Isthmus, and before the first World War many went to the States and did not return. At present numbers go to the United Kingdom in search of work.

Cancer Mortality Survey

The first table gives the average annual number of deaths from cancer, the crude death rate from cancer and the crude death rate from all causes by decades from 1887 to 1957. The table shows that for the last fifty years the cancer mortality has fluctuated between 53 and 74 per 100,000 inhabitants. It is reasonable also to infer that until the turn of the century a considerable number of cancer deaths were being labelled with some other diagnosis, unless one assumes that cancer mortality among coloured West Indians has increased considerably during the last three quarters of a century, which I think is unlikely to have been the case. Table I shows that the general death rate has fallen to less than a third of what it was in the late eighties of the last century, the fall being relatively most marked since the war. It will also be noticed that the Antigua crude death rate from cancer is one third of that for cancer in recent years in England and Wales.

As regards Table II, when allowance has been made for the wide fluctuation inevitable in data obtained from a small "universe", especially those noticed after the age of 65 years where figures are too small to justify inferences being drawn, female deaths have outnumbered male deaths per 1,000 at ages, especially during child-bearing years. This is in conformity with what has been met with elsewhere in the West Indies (Jamaica, 1952) and in coloured people in the United

TABLE I.—*Antigua*, 1887–1957. *Crude Death Rate from Cancer, in Decades*

Decade	Average annual number of deaths from cancer	Crude death rate from cancer per 100,000 (1)	Crude death rate all causes per 100,000 (2)	(1) as a percentage of (2)
1887–96	8	22	3181	0·7
1897–1906	13·8	41	3078	1·3
1907–16	16·2	53	2723	1·9
1917–26	16·0	54	2699	2·0
1927–36	24·9	74	1823	4·1
1937–46	27·1	72	1770	4·1
1947–56	32·2	69	1223	5·6
1957	34	63	924	6·8

Cancer crude death rate, England and Wales, 1954: 204 per 100,000.*
General crude death rate, England and Wales, 1954: 1190 per 100,000.
* Ministry of Health (1955).

States. In my opinion this disparity is only in small part occasioned by the emigration of the adult males already referred to By calculating the death rates per 1,000 at ages about census years, it has been possible to obtain data based on the actual numbers of persons of each sex in the island at those times. I have taken the average of the four years on each side of the census years, which enables me to approximate as much as possible to the decennial averages obtained in Europe and North America.

Table III gives an analysis of the 1410 deaths from cancer grouped according to the International List of Causes of Death, with comparable information from England and Wales.

Differences in the Distribution of Cancer as between the Black and White Races

Haslam (1926), Hoffman (1931), Delbet (1936), Des Ligneris (1936), Jonchère (1948) and Heller, Cutler and Haenzel (1955) have all drawn attention to the fact that the regional distribution of cancer in the negro differs considerably from that in the white race. The figures in Table III corroborate some of the evidence of certain of these authors, but not of others. I will consider the regions of the body where differences between the two races are shown up by a comparison of my data with those of others.

Cancer of the uterus

It is only in recent years that physicians and surgeons in Antigua have been specific as regards the situation in the uterus of a cancer. I have re-examined the records of the last 100 deaths from cancer of the uterus, i.e. all since 1948, and I found that 39 per cent were cancers of the cervix. This high relative mortality of cancer in the uterus in Antigua is in accord with what has been met with elsewhere in people of African descent; Heller *et al.* (1955) emphasise the high incidence of cancer of the cervix in the coloured person in the States. Hoffman (1931) found that in the American negro 44·7 per cent. of all cancers in the female were in the generative organs as compared with 24·4 per cent in the white race.

TABLE II.—*Cancer Mortality* 1887 *to* 1957; *Average Annual Death Rates per* 1,000 *at Ages in the Nine-year Periods Around the Census Years*

0–	5–	10–	15–	20–	25–	30–	35–	40–	45–	50–	55–	60–	65–	70–	75+	Census year
Males																
—	—	—	—	0·06	0·07	0·36	0·11	0·12	0·42	0·57	0·56	0·74	0·55	—	—	1891
0·06	—	—	0·07	0·22	—	0·18	—	0·98	0·48	1·90	1·40	1·68	2·75	3·33	5·55	1911
0·14	—	0·12	—	—	—	0·28	0·18	1·10	0·18	0·66	0·66	2·96	3·33	4·44	6·66	1921
0·04	0·05	0·05	0·07	—	0·21	0·09	0·16	0·28	0·95	1·96	6·03	5·18	7·15	3·85	9·35	1946
Females																
—	—	—	0·06	0·10	—	0·40	0·32	0·60	1·32	1·26	1·14	1·10	1·01	0·37	0·74	1891
0·06	—	—	—	0·07	0·21	0·30	0·60	1·32	1·30	2·94	2·64	2·20	2·52	4·40	1·1[illegible]	1911
—	0·09	—	—	—	—	0·55	0·50	0·84	0·66	3·20	1·76	5·94	3·36	4·96	3·85	1921
0·04	0·05	—	0·06	0·06	0·07	0·45	0·42	1·28	2·40	1·68	1·71	5·13	4·18	9·52	7·9[illegible]	1946
Persons																
—	—	—	0·03	0·09	0·03	0·36	0·25	0·42	0·84	0·96	0·88	0·98	0·88	0·28	0·56	1891
0·06	—	—	0·03	0·12	0·15	0·24	0·36	1·14	0·91	2·48	2·04	1·92	2·58	4·07	2·2[illegible]	1911
0·06	0·03	0·06	—	—	—	0·48	0·42	0·99	0·49	2·07	1·26	4·86	3·42	4·81	4·8[illegible]	1921
0·04	0·05	0·03	0·06	0·03	0·12	0·24	0·32	0·90	1·75	1·71	3·36	5·15	5·12	7·79	8·48	1946

In Antigua the figure is practically the same, 42 per cent. Higginson and Oettlé (1957) found cervical cancer to be nearly as common in the Bantu of Johannesburg as in the American negro. It has been maintained by some that the absence of circumcision in the male may be a factor in the aetiology of cervical cancer. It is true that very few males in Antigua are circumcised, but the greater likelihood in this Colony is that a more important part in the causation of cervical cancer is played by (a) the very high fertility rate of 140 to 145 per 1,000 females aged 15 to 44 years (Uttley, 1959, unpublished), and (b) early sexual intercourse with pregnancies usually following each other in quick succession in the teens and early twenties.

The material collected in Antigua is compared in Table IV with that from elsewhere in the West Indies, where the high proportional mortality as contrasted with that for England and Wales is well shown.

Cancer of the breast

On the other hand, the proportional mortality of cancer of the breast, which is much lower in the coloured person than among the women in England and Wales is shown in Table V.

Cancer at other sites

Primary cancer of the liver.—Smith and Elmes (1934) and Elmes and Baldwin (1947) writing about Nigeria, Jonchère (1948) dealing with French West Africa and Des Ligneris (1936) in South Africa have all emphasised the frequency of primary carcinoma of the liver in the negro. Table III shows that cancer of the liver is not uncommon in Antigua, but since it is not known whether the majority of these cancers are primary or secondary in nature, the information is of no value. On the other hand Kennaway (1944) maintains that in so far as the United States is concerned there is no greater liability for the negro to get primary cancer of the liver than the white person, and indicates that where the negro does in fact have a higher rate than the white there must be some intrinsic factor at work.

TABLE III.—*Analysis of the Last* 1410 *Deaths from Malignant Neoplasms in Antigua* 1887–1957, *with Corresponding Figures for England and Wales*, 1955

International statistical number	Malignant neoplasm of	Number		Proportion per 1000 total cancer deaths				
				Antigua		England and Wales*		
		Males	Females	Males	Females	Males	Females	
140–159	Total digestive system	244	322	624 (6·9)	316	433	439	
141	Tongue	8	1	20	1	8	3	
142	Salivary gland	2	0	5	0	—	—	143–148
144	Other parts of the mouth, unspecified	1	1	3	1	—	—	
145	Oral nasopharynx	1	2	3	2	18	10	
148	Pharynx, unspecified	9	6	23	6	—	—	
150	Oesophagus	16	13	41	13	28	22	
151	Stomach	101	135	258	132	166	143	
153	Large intestine except rectum	45	60	115	59	84	131	
154	Rectum	7	31	18	30	66	55	
155	Biliary passages	2	5	5	5	—	—	
156	Liver, unspecified	37	31	94	30	—	—	
157	Pancreas	4	4	10	4	38	38	
159	Unspecified digestive organs	11	33	28	32	—	—	
160–165	Total respiratory system	19	11	49 (5·2)	11	327	65	
161	Larynx	4	1	10	1	14	5	
162	Trachea, bronchi, lung (primary)	2	1	5	1	—	—	
163	Lung, bronchus, not specified whether primary or secondary	11	5	28	5	308	57	
164	Mediastinum	2	3	5	3	—	—	
165	Thoracic organs, secondary	0	1	0	1	—	—	
170–179	Total breast and genital organs	27	530	69	520	78	362	
170	Breast	0	90	0	88 (4·9)	2	197	
171	Cervix uteri	0	92	0	90	0	58	
173	Other parts of uterus	0	6	0	6 (2·7)	—	—	
174	Uterus, unspecified	0	321	0	315	0	32	172–174
175	Ovary fallopian tube, broad ligament	0	12	0	12	0	65	
176	Other female genital organs	0	9	0	9	—	—	
177	Prostate	11	0	28	0	69	0	
178	Testis	5	0	13	0	—	—	
179	Other unspecified male genital organs	11	0	28	0	2	0	
180–181	Total urinary systems	10	6	33	6	55	30	
180	Kidney	3	3	8	3	15	10	
181	Bladder	10	3	26	3	40	19	
191	Other malignant neoplasms of the skin	8	7	20	7	10	11	
192	Eye	1	1	3	1	—	—	
193	Brain	16	11	41	10	20	15	
194	Thyroid gland	2	2	5	2	2	6	
196	Bone, including jaw	20	16	51	16	9	0	
197	Connective tissue	6	8	15	8	—	—	
198	Secondary and unspecified neoplasms of lymph glands	0	1	0	1	—	—	
199	Unspecified	31	103	79	101	14	18	
201	Hodgkin's disease	1	0	3	0	—	—	
204	Leukaemia	3	1	8	1	52	46	200–205
	Total cancer	391	1019	1000	1000	1000	1000	

* From the Ministry of Health Reports (1955) (2).

The figures in brackets are the standard errors of those differences of the percentages between Antigua and England and Wales which are of statistical significance.

The standard errors of the differences of the percentages between the cancers of various sites in (1) Antiguans and (2) English people in Table III show that in four instances the differences are large enough to be statistically significant.

In the following sites the low relative mortality in Antigua as compared with that for England and Wales is statistically significant: in the respiratory system

TABLE IV.—*Cancer of the Uterus, Expressed as a Percentage of all Deaths from Cancer in Females of African Descent in the West Indies*

Colony	Years	Total cancer deaths in the female	Percentage due to cancer of the uterus
Leeward Islands—			
Antigua	1887–1957	1019	41
St. Kitts	1950–57	258	27
Montserrat	(Data not available in reports)		
Windward Islands—			
Grenada	1951	33	36
	1953	37	38
St. Vincent	(Data not available in reports)		
St. Lucia	1951–56 (excluding 1953)	43	40
Dominica	1950–54	55	39
Barbados	1949–54	672	34
Trinidad*	1947–55	2164	38
Jamaica	1952	407	28
British Guiana	1952–54	(Data not available in reports)	
England and Wales	1955	—	9†

* The Trinidad figures include East Indians, who constitute about one-third of the total population.

† In this and subsequent tables where data are quoted for England and Wales, they are from the Ministry of Health Reports (1955).

TABLE V.—*Cancer of the Breast, Expressed as a Percentage of all Cancer in Females of African Descent in the West Indies*

Colony	Years	Total cancer deaths in the female	Percentage due to cancer of the breast
Leeward Islands—			
Antigua	1887–1957	1019	9
	1950–57	162	9
St. Kitts	1950–57	258	11
Montserrat	(Data not available from records)		
Windward Islands—			
Grenada	1952	33	15
	1953	37	5
St. Vincent	(Data not available in reports)		
St. Lucia	1951–56 (excluding 1953)	43	5
Dominica	1950–54	55	20
Barbados	1949–54	672	11
Trinidad*	1947–55	2164	10
Jamaica	1952	407	12
British Guiana	(Data not available for the different races)		
England and Wales	1955	—	19·7

* The Trinidad figures include East Indians, who constitute one-third of the population.

and the female breast; whereas in the uterus and the digestive system as a whole the mortality is higher in Antigua.

Cancer of the stomach

In Table VI I have compared the relative mortality from cancer of the stomach in the two sexes in Antigua. It is much commoner per 1000 cancer deaths in the male than in the female.

The standard errors of the differences between the percentages of the West Indian and the English figures for cancer of the stomach are not statistically significant, but it will be noted that throughout the islands the mortality per 1,000 male cancers is approximately twice that per 1,000 female cancers. This should be compared with England and Wales where the corresponding figures are 16·6 and 14·3.

Average Age of Death in Women with Cancer of the Stomach as Compared with those Dying from Cancer of the Generative Organs

Gade (1931) raised an interesting point when he argued that not as many women as might have been expected die of cancer of the stomach because many cancer-disposed women have already succumbed to cancer of the uterus and ovary before they have had time to reach the age group in which cancer of the stomach normally occurs.

Gade was discussing Scandinavian women; Scandinavian food and environmental factors differ considerably from those prevailing in the West Indies;

TABLE VI.—*Cancer of the Stomach Expressed as a Proportion per* 100 *Total Cancer Deaths for Each Sex*

Colony	Years	Total cancer deaths			Number of deaths from cancer of the stomach		Gastric cancer deaths expressed as a percentage of total cancer deaths for each sex		Population in 1952 in thousands
		Male	Female	Total	Male	Female	Male (%)	Female (%)	
Leeward Islands—									
Antigua	1887–1957	391	1091	1410	101	135	26	13	48
St. Kitts	1950–57	112	258	370	32	39	29	15	51
Monserrat	No data available in reports								13
Windward Islands—									
Grenada	No data available in reports								82
St. Vincent									72
St. Lucia*	1951–56	27	43	70	16	8	59	19	83
Dominica	1950–55	54	55	109	26	5	48	9	57
Barbados	1951–54	228	429	657	91	88	40	21	219
Trinidad†	1947–55	1309	2164	3473	409	298	31	14	663
Jamaica	1952	322	407	729	105	67	33	17	1457
British Guiana	1952–54	—	—	120 (in negroes only)	No data available in reports				163 (negroes only)
England and Wales	1955	—	—	—	—	—	16·3	14·3	—

* Excluding 1953.

† The Trinidad figures include East Indians, who constitute one-third of the population.

however, I have compared the ages at death of women dying of the disease affecting their generative organs with the ages of those dying of cancer of the stomach. There can be little or no influence of one type of cancer on the other in so far as Antigua is concerned, because among the 199 women dying of cancer of the generative organs between 1920 and 1957, the average age of death was 49·3 years, (standard deviation of 13·0), and of the 72 who died in the same period from cancer of the stomach, the average age was 54·4 years, with a standard deviation of 13·2. The values of the standard deviations prevent us from assuming that there is any significant difference in the ages of death of the two conditions so far as Antigua is concerned.

Cancer Deaths in Relation to Deaths from all Causes

In Antigua the proportion of deaths from cancer to those from all causes is shown for each decade from 1887 to 1957 in Table I. the proportion has steadily risen over the years. Much of the increase is due to the improved death rates, especially in infancy, in recent decades, but it is also due to better diagnosis—to the salvaging of cancers from the terms such as "old age" mentioned in an earlier paragraph. It is also due to a larger number of people living into the cancer age groups in recent times. It may be that, if cancer incidence is proved to be on the increase in the negro, this factor also plays a part.

Cancer Death Rate in the West Indies

The following are the cancer death rates from neighbouring Caribbean islands. In general they corroborate the evidence collected in Antigua. In those instances where the death rate appears to be unduly low, one would suspect either that cancers are being missed or that not all deaths are medically certified, or both.

TABLE VII.—*Crude Death Rate from Cancer per* 100,000 *in the West Indian Coloured Population*

Leeward Islands—		
St. Kitts	1918–19	80
	1924–25	173
	1928–35	54
	1938–47	75
	1948–57	84
Montserrat	1947–49	80
Windward Islands—		
Grenada	1927–33	45
	1949–53	46
St. Lucia	1948–56	20
St. Vincent	1945–51	27
Barbados	1946–54	78
Trinidad*	1931–40	41
	1941–50	49
	1951–55	64
Jamaica†	1950	69
	1951	66
	1952	71
British Guiana (negro population only)	1932–38	45
	1952–54	23

* One-third of the population are East Indians.
† About one-third of all deaths are not medically certified.

Varieties of Neoplasm

The Antigua death reports do not help much in differentiating the various types of neoplasm other than carcinoma and sarcoma. An analysis of the last 320 deaths from malignant growth, all having occurred since 1948, reveals that there were six, or less than 2 per cent, due to sarcomas. This compares with a ratio of one to seventeen met with in Europe (Vint, 1935). Table VIII shows the rates that have been met with in the coloured people:

TABLE VIII.—*Cancer death Rates in Coloured People*

Author	Country	Per cent due to carcinomas	Per cent due to sarcomas	Carcinoma : sarcoma ratio	Remarks
Hartz (1950)	Curaçao, N.W.I.	10·2	1·4	7 : 1	2000 autopsies.
Jonchère (1948)	French West Africa	69	19·5	3·5 : 1	Microscope examination of 615 tumours.
Choisser (1929)	Haiti	3·4	0·5	7 : 1	700 consecutive autopsies of which 27 were malignant tumours.
Smith and Elmes (1934)	Nigeria	45	44	1 : 1	445 malignant tumours.
Vint (1935)	Kenya	51	34	1·5 : 1	561 malignant tumours.
This paper	Antigua	98·1	1·9	50 : 1	320 malignant tumours.

The carcinoma-sarcoma ratio as met with in Antigua is very different from what has been met with elsewhere in Africans or people of Africa descent, and I am not prepared to offer a reason for it.

Sex Ratio of Malignant Neoplasms

Table IX shows the sex ratio of cancers in so far as the West Indies is concerned; cancer deaths occur about twice as frequently in females as in males throughout the area.

In many of the territories adult females out-number adult males. This is nowhere so marked as in Antigua where the ratio is 1,000 to 754, but this disparity in the numbers of the sexes is not sufficient materially to affect the general conclusion about the sex ratio of cancer mortality.

TABLE IX.—*Sex Incidence of Deaths from Cancer in the British Caribbean*

Territory	Years	Males, number and percentage	Females, number and percentage
Antigua	1887–1957	319 (27·7)	1019 (72·3)
St. Kitts	1949–57	112 (30·3)	258 (69·7)
Montserrat	(Reports do not record the sex)		
Grenada	1949–53	61 (32·4)	127 (67·6)
St. Vincent	(Reports do not record the sex)		
St. Lucia	1948–56 (excluding 1953)	49 (36·6)	85 (63·4)
Dominica	1950–54	54 (49·1)	55 (50·9)
Trinidad	1947–55	1309 (37·7)	2164 (62·3)
Jamaica	1952	322 (44·1)	407 (55·9)
Barbados	1948–54	373 (32·0)	796 (68·0)
British Guiana	(Data not available in reports)		

SUMMARY AND CONCLUSIONS

A survey of all death certificates in Antigua has been made during an investigation of the cancer death rate in the coloured inhabitants of the island. It revealed that 1410 deaths since 1887 have been due to malignant disease, which when calculated as deaths per 100,000 living showed a rate of about one third of that prevailing in England and Wales at present.

Cancer has a higher mortality among females per 1,000 at ages than among males in Antigua, especially during the later child-bearing years.

Cancer of the uterus and of the digestive system is commoner per 1,000 cancer deaths in Antigua than in England and Wales, whereas in the respiratory system and in the female breast it is not so common in the Colony.

A comparison of the data from Antigua with similar material from adjacent Colonies, although the latter is scanty and incomplete, indicates that there is little difference between the total cancer death rate, the death rates from cancer of the stomach, uterus and of the breast severally in Antigua on the one hand, and in the neighbouring territories on the other.

The sex ratio of cancer deaths is two females to one male ; this appears to be the ratio throughout the British West Indies.

The carcinoma sarcoma ratio in Antigua, 50 to 1, is very much higher than other published reports of the ratio in the negro.

The death returns do not suggest that cancer has been increasing much in this part of the West Indies during the last half century, though it is possible that there may have been an increase in late middle life.

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REFERENCES

Choisser, R. M.—(1929) *Nav. med. Bull., Wash.*, **27**, 551.
Delbet, P.—(1936) *Bull. Acad. Méd., Paris*, **115**, 483.
Des Ligneris, M.—(1936) *S. Afr. med. J.*, **10**, 478.
Elmes, B. G. T. and Baldwin, R. B. T.—(1947) *Ann. trop. Med. Parasit.*, **41**, 321.
Gade, F. G.—(1931) *Norsk. Mag. Laegevidensk.*, **92**, 593.
Hartz, P. H.—(1950) *Docum. neerl. indones. Morb trop*, **2**, 161.
Haslam, F. J. C.—(1926) *J. Hyg.*, **25**, 227.
Heller, J. R., Cutler, S. J. and Haenzel, W. M.—(1955) *J. Amer. med. Ass.*, **159**, 1628.
Higginson, J. and Oettlé, A. G.—(1957) *Acta Un. int. Cancr.*, **13**, 949.
Hoffman, F. L.—(1931) *Amer. J. Surg.*, **14**, 229.
Jonchère, H.—(1948) *Bull. méd. A.O.F.*, **5**, 247.
Kennaway. E. L.—(1944) *Cancer Res.*, **4**, 571.
Ministry of Health.—(1955) The report of the Ministry of Health, Part 2. On the state of public health. London.
Idem.—(1955) (2) The report of the Ministry of Health, Part 2. On the state of public health. London, p. 114.
Smith, E. C. and Elmes, B. G. T.—(1934) *Ann. trop. Med. Parasit.*, **28**, 461.
Vint, F. W.—(1935) *Lancet*, ii, 628.
Wilcocks, C.—(1932) *E. Afr. med. J.*, **9**, 88.
Barbados.—Annual Reports of the Registrar on the Vital Statistics. Bridgetown.

BRITISH GUIANA.—Annual Reports of the Registrar General. Georgetown.
DOMINICA.—Reports of the Medical and Sanitary Departments. Roseau.
GRENADA.—Reports and General Abstracts of the Registrar General. St. Georges.
JAMAICA.—Annual Report of the Registrar General for the year 1952. Kingston, p. 36.
MONTSERRAT.—Annual Reports of the Medical Department. Plymouth.
ST. KITTS.—Annual Reports of the Medical Department. Basseterre.
ST. LUCIA.—Annual Reports of the Registrar General (Medical Department). Castries.
ST. VINCENT.—Annual Administration Reports of the Colony of St. Vincent. Kingstown.
TRINIDAD.—Central Statistical Office. Population and Vital Statistics. Annual Report for the year 1955. Port of Spain.

SOME PILOT OBSERVATIONS ON OESOPHAGEAL CARCINOMA DEATHS IN MENTAL PATIENTS

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DURING an investigation for another purpose conducted on the records of patients at Friern Mental Hospital for the years 1949–1956, the author noticed that, out of 144 carcinoma deaths, 12 were oesophageal. As it seemed possible that these might indicate a relative excess of oesophageal cancer, it was thought worthwhile to compare the proportion of cancer deaths due to oesophagus with the corresponding figures for the population of England and Wales. In the accompanying Table I, which has been calculated from Table 17 of the Registrar General's Statistical Review of England and Wales (1954), it will be seen that about 2½ per cent of the total cancer deaths for the country as a whole are oesophageal. Of 144 cancer deaths at Friern in the period 1949 to 1956, 12 were oesophageal, i.e. 8·3 per cent. Of these cancer deaths 56 were in males, of whom 5 died from oesophageal carcinoma, i.e. 9 per cent. The proportion of all cancer deaths in men for England and Wales that was due to oesophageal carcinoma was 2·9 per cent. Out of 88 carcinoma deaths in women in Friern during 1949 to 1956, 7 were due to oesophageal carcinoma, i.e. 8 per cent. The corresponding figure for women in England and Wales who died from oesophageal carcinoma was 2 per cent. It follows that the difference in proportion of cancer deaths due to oesophagus between Friern and England and Wales is not due to sex differences, as the Friern excess is found in men and women. Examination of Table I also shows the proportion of carcinoma deaths recorded as due to oesophagus for different age groups in men and women. All the oesophageal cancer deaths in Friern were in-patients aged 60 or over. It will be seen that the national figures range from about 2·4 to 4·5 per cent of all cancer deaths in each age group in males, and from 1·8 to 3 per cent for the relevant age groups in females. The corresponding figures for Friern demonstrate a relative oesophageal excess in each age group.

The difference in age/sex composition of the Friern cancer death patients from the England and Wales deaths does not then account for the excessive proportion recorded as oesophageal in this series, since it occurs in each age/sex group. Obviously there is no information as to whether or not there is an absolute increase in oesophageal carcinoma in Friern because the relative increase could be due to either an absolute increase in oesophagus deaths or a relatively small number of all other cancer deaths. This cannot be decided at the moment, but the analysis that follows would not be factually invalid if, merely to allow the investigation to proceed, we postulate that there is an excess of oesophageal cancer among the Friern patients which requires explanation. We may then ask the following question :— " Is there any evidence to suggest that there may be an aetiological factor in oesophageal carcinoma special to the mental hospital patient or his

TABLE I

	MEN						WOMEN					
	*Carcinoma deaths		*Oesophagus carcinoma deaths		Oesophagus deaths as % of all carcinoma deaths in each age group in men		*Carcinoma deaths		*Oesophagus carcinoma deaths		Oesophagus deaths as % of all carcinoma deaths in each age group in women	
Age groups	England and Wales (No.)	Friern (No.)	England and Wales (No.)	Friern (No.)	England and Wales (%)	Friern (%)	Age group	England and Wales (No.)	Friern (No.)	England and Wales (No.)	Friern (No.)	England and Wales (%)	Friern (%)
25—	259	..	5	..	1·9	..	25—	222	..	2	..	0·9	..
30—	364	2	2	..	0·55	..	30—	474	..	4	..	0·84	..
35—	632	..	6	..	0·95	..	35—	781	..	5	..	0·64	..
40—	1,254	..	21	..	1·7	..	40—	1,602	2	9	..	0·55	..
45—	2,406	1	24	..	1·0	..	45—	2,585	4	31	..	1·2	..
50—	4,012	3	71	..	1·7	..	50—	3,424	4	53	..	1·5	..
55—	5,265	6	102	..	1·9	..	55—	4,318	4	76	..	1·7	..
60—	6,570	11	160	1	2·4	9·1	60—	5,196	14	94	1	1·8	7·1
65—	7,443	12	222	1	2·9	8·3	65—	5,937	15	117	4	1·9	26·7
70—	7,305	9	263	2	3·6	22·2	70—	6,256	21	159	..	2·6	..
75—	6,143	9	265	1	4·3	11·1	75—	5,838	16	153	..	2·6	..
80—	3,159	1	144	..	4·5	..	80—	3,647	4	99	1	2·8	25·0
85+	1,183	2	50	..	4·2	..	85+	2,032	4	63	1	3·0	25·0
All ages	46,773	56	1,335	5	2·9	8·9	All ages	42,907	88	865	7	2·0	7·9

* The figures for England and Wales are taken from Table 17 of the Registrar General's Statistical Review (1954). The figures for Friern are taken from Death Certificate Records 1949-56, i.e. with 1953 as the central year.

	Total carcinoma deaths		Total oesophagus carcinoma deaths	
	England and Wales	Friern	England and Wales	Friern
Men	46,773	56	1,335	5
Women	42,907	88	865	7
	89,680	144	2,200	12

environment?" *If* this were so it might be expected that the proportion of cancer deaths due to oesophagus might vary according to the number of years spent in the hospital. Accordingly, the cancer deaths amongst this mental hospital's inmates for 1949–1956 were divided into two groups :—

(*a*) people who had been in Friern less than ten years before their death from carcinoma and

(*b*) those who had been inmates more than ten years before their death from carcinoma.

It was then found that, out of 89 persons who had been in Friern less than ten years before their death from carcinoma, 3 died from carcinoma of the oesophagus, i.e. 3·4 per cent, which is little different from the national proportion of 2·5 per cent amongst the general population of all people who die from oesophageal cancer. Out of 55 persons who were in Friern ten years or more before their death from carcinoma, 9 died from carcinoma of the oesophagus, i.e. 16 per cent. This is apparently a grossly excessive proportion over what would be expected if duration of stay were not relevant. Of course the longer a patient is in hospital the older he will be and Appendix A shows the average ages of " long-stay " male cancer death cases to be 69, while the average age of the " short-stay " males who died from cancer was 65. The average age of the " long-stay " females was 69 and of the " short-stay " females 67. It is clear from examining the figures in Table I that age differences in the " long-stay " compared with the " short-stay " groups do not account for the gross increase in the oesophageal carcinoma deaths in the " long-stay " groups.

Effect of duration of stay on male cancer deaths

Out of 40 men in Friern less than ten years before their death from carcinoma, 1 died from carcinoma of the oesophagus = 2·5 per cent.

Out of 16 men in Friern ten years or more before their death from carcinoma, 4 died from carcinoma of the oesophagus = 25 per cent. The male average was 9 per cent.

Effect of duration of stay on female cancer deaths

Out of 49 women in Friern less than ten years before their death from carcinoma, 2 died from carcinoma of the oesophagus = 4 per cent.

Out of 39 women in Friern ten years or more before their death from carcinoma, 5 died from carcinoma of the oesophagus = 13 per cent. The female average was 8 per cent.

CONCLUSION

It appears from this limited study that there is a relative increase in the proportion of cancer deaths due to oesophagus in Friern Hospital patients compared with the proportion found in the general population of England and Wales. This relative excess is not apparently explained by the differences in age and sex composition of the cancer death cases in the two groups.

The proportion, 3·4 per cent of cancer deaths due to oesophagus in " short-stay " patients (less than ten years) is less than the hospital average of 8·3 per cent.

The proportion of cancer deaths due to oesophagus in " long-stay " patients, 16 per cent, is much more than the hospital average of 8·3 per cent.

These differences are not apparently due to the increased ages of the "long-stay" patients and hold for both males and females. It appears that there may be an aetiological factor in the very chronic mental patient, or, more likely, in his environment, that favours the development of oesophageal carcinoma.

The next step could be to examine casepapers of oesophageal cancer patients and controls to see whether there are any clues to what this factor(s) may be, e.g. long term consumption of paraldehyde, lubricant for tube-feeding, etc.

I am grateful to Dr. A. C. Dalzell for allowing me access to his records. Thanks are due to the staff of the Mass X-Ray Service for assistance, especially Miss J. Strudwick and Mrs. M. Levett.

This paper gives an account of part of some work carried out for and successful in the Research Report Competition of the South West Metropolitan Regional Hospital Board for 1958.

APPENDIX A

Friern Hospital

Deaths from Carcinoma, from 1949–July, 1956

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TRIETHYLENE THIOPHOSPHORAMIDE IN THE TREATMENT OF PREVIOUSLY HYPOPHYSECTOMISED PATIENTS WITH CARCINOMA OF THE BREAST

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PATIENTS who have undergone complete hypophysectomy in the treatment of carcinoma of the breast hardly ever show subsequent improvement when treated with hormones such as androgens, oestrogens or cortisone analogues. This clinical observation supports the conclusion, in most experimental animals, that after hypophysectomy there is little or no response by breast tissue to stimulation with oestrogens. Apart from general care and analgesics the palliation of patients with advanced carcinoma of the breast uncontrolled by pituitary ablation is therefore limited to radiotherapy, the cytotoxic drugs and possibly 3-methylcholanthrene (Huggins and McCarthy, 1957). The effect of hypophysectomy, though satisfactory in over 50 per cent of patients, seldom lasts longer than three to four years (Luft *et al.*, 1958 ; Ray and Pearson, 1956 ; Baron *et al.*, 1958). Very often the general condition of these patients is surprisingly good at the time of relapse or when it becomes clear that hypophysectomy has not been beneficial. Attempts at further treatment are sometimes justified. Because hormones are ineffective and in the majority of cases radiotherapy is contra-indicated, we have studied the effect of the aklylating agent triethylene thiophosphoramide (thiotepa). Shay and his colleagues (Shay *et al.*, 1953 ; Shay and Sun, 1955) first suggested that thiotepa might be of benefit to patients with metastatic carcinoma of the breast. Subsequently Bateman and her colleagues (Bateman, 1955 ; Bateman and Larsen, 1956) reported improvement in up to 85 per cent of patients so treated. It appears that thiotepa is relatively more effective in the treatment of mammary and ovarian carcinoma than in lymphomas such as Hodgkin's disease or leukaemias.

METHODS

Thiotepa was dissolved in water in concentrations of 1 mg. to 5 mg. per ml., sterilised by Seitz filtration, stored in a refrigerator and used within three weeks of preparation. It was given by intramuscular injection, the usual plan being to give 5 mg. daily for five days in the first week with a similar dose in the second week if the white cells and platelet counts were unaffected. Treatment was continued with 5 mg. I.M.I. two or three times a week for as long as possible, reducing to 3 mg. if the white count showed signs of falling. No treatment was given if the W.B.C.s were 3,000 c.mm. or under or the platelets below 140,000 c.mm. Pain after injection was not usually severe but persisted for about half an hour in some patients. The intramuscular route was chosen because of the ease of

administration, particularly in out-patients, but in one instance injections were also given into a tumour mass and three patients had intrapleural injections of 20 mg. of thiotepa.

RESULTS

Eighteen women who had previously undergone surgical hypophysectomy, combined with radioactive gold seed implantation, have been treated for periods of up to 56 weeks. Eight had shown objective improvement after the operation. Treatment with thiotepa was continued for six weeks or more in all but one patient whose white cell and platelet counts fell precipitously after three weeks and in whom there were signs of leuko-erythroblastic anaemia. The total dose of thiotepa ranged from 65 to 520 mg.

Objective improvement such as reduction in the size of lymph nodes, cutaneous and hepatic metastases or locally recurrent tumour, was observed in 10 of the 18 patients (56 per cent). In seven (39 per cent) objective improvement was maintained for eight weeks or more, but in two improvement was transient and lasted for between four and six weeks only. Although objective and subjective improvement were usually related, in one patient (Case 18) there was considerable reduction in the size of supra-clavicular and axillary lymph nodes for more than two months without subjective benefit. A summary of some of the clinical findings, dosage and toxic effects is given in Table I. Bone pain from metastases has been relieved in four patients, obstructive jaundice in two, severe paroxysmal auricular fibrillation caused by mediastinal and pericardial involvement in another, and peripheral blood changes indicating leuko-erythroblastic anaemia have improved in two instances. The duration of improvement has been variable. Four have maintained objective improvement for over six months, the longest period of remission being 13 months in a woman with cutaneous metastases, the total dose of drug in these women being 280, 235, 390 and 520 mg. given intermittently. In two patients the paradox was seen of certain metastases improving whilst new ones appeared elsewhere, a fairly common occurrence in patients with hormone sensitive tumours.

It is possibly of significance that the four women who showed the most improvement had all previously responded well to hypophysectomy for between six months and two years before receiving thiotepa, and it may be that their tumours were intrinsically more responsive to any type of treatment. The histological appearances were not related to the therapeutic response.

Toxic Effects

Apart from pain at the site of injection the only troublesome side effect was haematopoietic depression. The white count fell to below 3,000 c.m. in five patients, the two lowest being 1,800 c.mm. after a total dose of 155 mg., and 1,900 per c.mm. after 520 mg., the granulocytes being more severely depressed than the lymphocytes. In none of the women in this series were the lymphocytes selectively involved. Recovery from mild granulocytopenia usually occurred within 10–21 days of stopping treatment. In Case 2 the white cell count rose from 1,900 to 6,200 per c.mm., with a normal differential by the fifteenth day. A significant fall in platelet count to 120,000 c.mm. or under was observed in five patients, the lowest being 86,000 after 155 mg. thiotepa given over a period of 11 weeks. No

TABLE I.—*Summary of Clinical and Haematological Findings Before and After Treatment with Thiotepa*

Case	Age (years)	Site of disease	Thiotepa: Total dose (mg.)	Thiotepa: Duration (weeks)	Initial blood count: Hb. (G/100 ml.)	Initial blood count: W.B.C. (per c.mm.)	Initial blood count: Platelets (per c.mm.)	Blood count during treatment: Hb. (G/100 ml.)	Blood count during treatment: W.B.C. (per c.mm.)	Blood count during treatment: Platelets (per c.mm.)	Clinical response: Objective	Clinical response: Subjective	Clinical response: Duration (weeks)	Remarks
1	53	Liver Lungs Bones	390	36	10·4	8,100	239,000	12·4	4,900	118,000	+	+	32+	Jaundice cleared; L.E. anaemia improved.
2	58	Local recurrence Pleura	520	56	13·6	6,300	346,000	10·8	1,900	112,000	+	+	56+	Pectoral metastases regressed.
3	47	Lungs Lymph nodes	125	12	13·4	10,400	232,000	13·8	8,600	256,000	+	+	10	Cough relieved; nodes smaller
4	38	Bones Brain	95	6	11·4	5,200	282,000	10·8	6,400	320,000	+	+	5	Less drowsy; headache relieved.
5	34	Breast Pleura Bones	100	8	14·1	6,400	312,000	13·4	6,800	289,000	0	0	0	No response.
6	66	Local recurrence Lymph nodes Lungs	90	6	12·2	9,600	256,000	11·8	8,200	286,000	0	0	0	No response.
7	44	Lungs Bones	95	6	11·0	9,400	220,000	12·4	6,600	206,000	0	0	0	No response.
8	58	Lymph nodes Pleura Bones	120	10	15·4	12,600	236,000	15·6	5,200	174,000	+	0	8	Fungating nodes regressed; died of pulmonary embolus.
9	48	Pleura Pericardium Lymph nodes	185	14	9·6	7,100	348,000	10·5	4,400	188,000	+	+	12+	Lymph nodes and pleural effusion smaller. L-E anaemia improved.
10	62	Breast Lymph nodes Dermatomyositis	170	10	10·8	6,400	242,000	8·6	5,200	135,000	0	0	0	No response.
11	64	Breast Pleura Lymph nodes	125	8	12·6	5,100	362,000	11·8	2,800	233,000	0	0	0	No response.
12	62	Lymph nodes Liver	280	28	14·5	8,300	282,000	12·4	3,200	182,000	+	+	26+	Jaundice cleared. Nodes and liver smaller.
13	52	Skin Pleura	325	36	14·2	6,800	274,000	9·4	4,000	136,000	+	+	30+	Skin metastases disappeared.
14	36	Pleura Pericardium Lymph nodes Liver	110	7	10·1	5,600	212,000	9·2	2,800	116,000	+	+	4	Paroxysmal tachycardia relieved; lymph nodes smaller, liver larger.
15	56	Lungs Pleura	110	8	11·6	9,200	244,000	10·8	5,600	256,000	0	0	0	No response.
16	66	Bones Liver	65	3	10·8	6,400	206,000	10·8	1,200	66,000	0	0	0	L-E anaemia; treatment discontinued.
17	43	Breast Liver Bones	130	8	13·2	8,800	268,000	12·8	4,900	186,000	0	0	0	No response.
18	54	Lymph nodes Liver	155	11	14·2	6,900	240,000	13·9	1,800	86,000	+	0	8	Lymph nodes smaller; leukopenia; treatment stopped.

haemorrhagic complications developed. The most rapid and severe bone marrow depression seems most likely to occur when there are leuko-erythroblastic changes but this is not necessarily a contraindication to treatment since improvement may result. No significant fall in haemoglobin levels could be attributed to treatment during the time these patients came under observation.

DISCUSSION

In this group of patients in whom all other forms of treatment from mastectomy to hypophysectomy had failed to control the tumour, thiotepa has led to significant objective improvement in about one third for over six months at a time when rapid deterioration was anticipated. The prolongation of life and relief of symptoms has sometimes been significant and worthwhile and the unpleasant consequence of treatment minimal. Although the degree of improvement to be expected in these patients is relatively small, the disappearance of obstructive jaundice and control of troublesome metastases has allowed two women who had previously been confined to bed to return to house work. Constant supervision, regular blood counts and individual adjustment of dosage are of course essential, and the improvement is no more than temporary, but the response compares favourably with the effect of analgesics and the usual care given to the dying patient. In any case the use of thiotepa does not exclude the administration of morphia or cocaine as the comfort of the patient demands. Batemen and Larsen (1956) claimed that 80 per cent of their patients with breast cancer were improved even to the extent of recalcification of bone lesions. They emphasised that a slow response was not unusual and that, as with hormones, maintenance treatment was desirable. On the other hand Ultmann *et al.* (1957) found intravenous thiotepa to be effective in the treatment of ovarian carcinoma, but none of the patients with mammary cancer improved. There was a high incidence of haematological depression, 47 per cent of patients developing an abnormal blood picture while 17 per cent had clinical manifestations of bleeding or toxicity. Leonard, Israels and Wilkinson (1956) also encountered a higher incidence of side effects in patients with a variety of lymphomas who were given doses such as 25 mg. per injection. It seems probable that the use of relatively small doses given intramuscularly for as long as possible is less toxic, more effective and to be preferred to more intensive treatment intravenously. Although the value of triethylene thiophosphoramide is limited, a good response in some of these hypophysectomised women with advanced carcinoma of the breast suggests that such treatment should be tried in certain patients when other more effective remedies have failed.

SUMMARY

Eighteen women with advanced metastatic carcinoma of the breast have been treated with intramuscular triethylene thiophosphoramide in doses of up to a total of 560 mg. for periods of up to 56 weeks. Objective improvement was noted in 10 patients (56 per cent), in seven of whom improvement was maintained for eight weeks or more with a maximum of 56 weeks. It is considered that prolonged intramuscular treatment with thiotepa is worthwhile in patients who have failed to respond to, or relapsed after, hormone therapy or endocrine surgery.

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REFERENCES

BARON, D. N., GURLING, K. J. AND RADLEY SMITH, E. J.—(1958) *Brit. J. Surg.*, **45**, 593.
BATEMAN, J. C.—(1955) *New Engl. J. Med.*, **252**, 879.
Idem AND LARSEN, N. J.—(1956) *J. Amer. geriat. Soc.*, **4**, 341.
HUGGINS, C. and MCCARTHY, J. D.—(1957) *Cancer Res.*, **17**, 1028—32.
LEONARD, B. J., ISRAELS, M. C. G. AND WILKINSON, J. F.—(1956) *Lancet* ii, 1017.
LUFT, R., OLIVECRONA, H., IKKOS, D., NILSSON, L. B. AND MOSSBERG, H.—(1958) in ' Endocrine Aspects of Breast Cancer ', Ed. Currie, A. R. Edinburgh (E. & S. Livingstone), p. 27.
RAY, B. S. AND PEARSON, O. H.—(1956) *Ann. Surg.*, **144**, 394.
SHAY, H. AND SUN, D. H.—(1955) *Cancer*, **8**, 498.
Idem, ZARAFONETIS, C., SMITH, N., WOLDOW, I. and SUN, D. H.—(1953) *Arch. intern. Med.*, **92**, 628.
ULTMANN, J. E., HYMAN, G. A., CRANDALL, C., NAUJOKS, H. AND GELLHORN, A.—(1957) *Cancer*, **10**, 902.

LYMPH NODE AND LIVER BIOPSY IN THE MYELOPROLIFERATIVE DISORDERS

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THE syndrome of anaemia, splenomegaly, leuco-erythroblastosis of the peripheral blood and hypocellularity of bone marrow aspirates is a well-recognised entity. The importance of bone biopsy in establishing a diagnosis of myelofibrosis in a number of these cases has been shown in recently reported case series and without it this diagnosis cannot be fully proven (Hutt, Pinniger and Wetherley-Mein, 1953; Korst, Clatanoff and Schilling, 1956). However, this condition or a related myeloproliferative disorder must be seriously suspected before a bone biopsy is decided upon and the very variable clinical presentation of the syndrome frequently results in other diagnoses being made first.

The splenic enlargement may be only slight and loss of weight and weakness are often the only symptoms, the clinical picture suggesting malignant cachexia. Lymphadenopathy is occasionally a presenting feature, and a primary disorder of lymph nodes may be suspected. In the peripheral blood, precursor cells of the red and white cell series may not be found (Tudhope, 1937), particularly in the early stages (Taylor and Simpson, 1950) or they may be present intermittently (McMichael and Mcnee, 1936). Anaemia is not necessarily severe. Some cases may present as a haemolytic anaemia or as a haemorrhagic disorder with platelet abnormalities. The findings on bone marrow aspiration are frequently equivocal and a proportion of patients yield a moderately cellular specimen showing normal marrow elements or prominent normoblastic red cell maturation (Leonard, Israëls and Wilkinson, 1956). Such appearances may draw attention away from the diagnosis of myelofibrosis or related disorder and the danger of drawing firm conclusions from a single marrow aspirate has been stressed (Heller, Lewisoh and Palin, 1947). Finally, prominent hepatomegaly or abnormalities of liver function may be the principal clinical findings and primary liver diseases may be suspected. The coexistence of cirrhosis and myelofibrosis is discussed by Wyatt and Sommers, (1950).

Initial diagnosis such as chronic myeloid leukaemia, " refractory " anaemia, or " malignant reticulosis " may result in therapy such as irradiation which may be harmful to the patient. When dealing with this obscure clinical group, any finding which suggests the possibility of a myeloproliferative disorder and indicates the need for bone biopsy examination will therefore be of importance. In a series of 20 cases of proven myelofibrosis or related disorder, four cases showed changes in either the lymph glands or the liver biopsy specimen which have helped in establishing the correct diagnosis. In all cases the diagnosis was finally confirmed by bone biopsy or autopsy studies. Their clinical and pathological features will be briefly discussed.

Case Reports

Case 1

A woman of 30 suffered from occasional attacks of grand mal and had been treated by phenobarbitone and hydantoinates for several years. For two years she had been troubled by occasional transient dragging pains in the left flank, sometimes associated with sweating. In childhood she had rheumatic fever and chorea. On admission for investigation she was pale, the signs of mitral stenosis were present, the spleen was enlarged almost to the level of the umbilicus and the liver was just palpable.

Investigations.—A blood count showed haemoglobin 12·5 g., white cells 5,700 per c. mm. with normal differential count. The bleeding time, clotting time and platelet count were normal. The red cells showed considerable anisocytosis with an occasional macrocyte. Investigation of the renal tract showed no abnormalities. Marrow aspiration yielded a poorly cellular specimen. Scanty normal marrow elements were present.

The findings were suggestive of myelofibrosis and rib biopsy was carried out. As splintering occurred during removal it was thought that this material might prove unsatisfactory, so liver biopsy was undertaken a few days later. The liver biopsy showed many multinucleate cells within the sinusoids (Fig. 1). These cells had a pale homogenous acidophilic cytoplasm; some resembled mature megakaryocytes. A very occasional primitive myeloblast-like cell was present within the sinusoids. The bony material showed several areas of marrow. These contained groups of primitive myeloid cells among which were a number of bizarre megakaryocyte-like cells. Reticulum cells and fibroblast-like cells were present, and there was a moderate diffuse increase in reticulin. No normal erythropoietic tissue was seen, nor was there leucopoietic differentiation. The appearances were those of myelofibrosis.

Progress.—The abdominal pains were attributed to the splenic enlargement. She improved clinically and was discharged to another district, the diagnosis being "megakaryocytic myelosis and early myelofibrosis". She died suddenly at home shortly afterwards and no autopsy was obtained.

Case 2

A man of 52 had complained for 7 years of occasional pains in the abdomen after meals, and bouts of vomiting. Gastric ulceration was demonstrated radiologically. He had been a coal miner for 21 years, and chest X-rays revealed diffuse reticulation with early lung fibrosis. He had recently complained of pains at the left costal margin, and examination showed the spleen to be just palpable. On admission to hospital for investigation of the splenomegaly, the blood pressure was 120/80 mm. Hg.

Investigations.—A blood count showed haemoglobin 16·6 g., red cells 6,040,000 per c. mm., white cells 26,100 per c. mm. The blood film revealed considerable polychromasia of the red cells with an occasional myelocyte and normoblast. Sternal marrow aspiration yielded an active cellular marrow, with hyperplasia of all marrow elements. A diagnosis of polycythaemia vera was made.

Progress.—During the next 5 years his abdominal symptoms were well controlled by symptomatic measures. He then complained of generalised pruritus and of impotence. The splenomegaly became more obvious, and he began to lose

weight. He was paler, and dyspnoeic on exertion. Attacks of bronchitis with purulent sputum were frequent. Blood counts during this period showed an initial increase in values (haemoglobin 20·1 g., red cells 6,550,000 per c. mm.) followed by a steady fall in haemoglobin to 11·8 g. and 9·8 g. The white cell counts varied from 18,000 to 42,000 per c. mm. with small numbers of metamyelocytes (1 to 7 per cent) and of normoblasts. The platelet count remained normal. He received no irradiation or other specific treatment during the polycythaemic phase of illness.

Marrow aspirations 1, 3 and 5 years after his first admission yielded poorly cellular specimens, that at three years showing normal elements with a preponderance of cells of the myeloid series (myeloid : erythroid ratio, 8 : 1), while that at 5 years consisted almost entirely of peripheral blood.

Serial chest X-rays showed gradually increasing density of shadowing of the lung fields. Recent enlargement of the liver was noted. The development of hepatomegaly and of marrow failure following his previously polycythaemic state led to rib biopsy and liver biopsy.

Rib biopsy showed a moderately cellular marrow, the predominant cells resembling primitive myeloblasts. Some developing forms and polymorphs were present. Very many megakaryocytes in various stages of maturation were present. Erythropoiesis was inconspicuous. The reticulin pattern showed a general diffuse increase with focal areas of more closely enmeshed fibrils. Liver biopsy showed essentially normal parenchyma. The sinusoids contained diffuse and focal areas of myeloid tissue. A few of the focal collections showed the pleomorphic appearances of haemopoietic tissue with normoblastic development. The diffusely distributed cells were largely primitive myeloid cells, multinucleate cells being inconspicuous (Fig. 2).

He developed a persistent sinus of the chest wall, the discharge from which yielded tubercle bacilli on culture. Repeated sputum examinations throughout his illness had been negative for tuberculosis. He was discharged and died suddenly at home soon afterwards, and no autopsy was performed.

Comment

In Case 1 the chief symptom of left-sided abdominal pains was attributed to the enlarging spleen. The myeloproliferative process had been otherwise symptomless. Suggestive findings were the moderate anisocytosis of the red cells despite the reasonably high level of haemoglobin, and the poorly cellular marrow aspirate. Liver biopsy established the presence of megakaryocytic myelosis, bone biopsy confirming the presence of myelofibrosis.

Case 2 showed clinical and haematological evidence of polycythaemia vera, began to lose weight and became anaemic. He received no radiotherapy or other specific treatment. The liver became enlarged. Developing marrow failure was suspected and serial marrow aspirations supported this view. Rib biopsy was carried out to confirm this diagnosis, liver biopsy providing additional information as to the nature of the hepatomegaly and the presence of visceral involvement in the myeloproliferative process.

The case illustrates the transition from a polycythaemic process to myelofibrosis with myeloid metaplasia in the absence of specific treatment ; the coexistence of a tuberculous sinus is a further point of interest ; however no aetiological

relationship between the tuberculosis and the haematological changes could be established.

Case 3

A woman aged 62 complained of weakness and attacks of sweating for 2 months, with shortness of breath and loss of weight. A small cut on the lip became infected and throat ulceration developed. On examination, there was extensive ulceration and inflammation of the palate and fauces. The liver was much enlarged and the spleen very slightly enlarged clinically. The axillary, cervical and inguinal glands were also moderately enlarged and soft.

Investigation and progress.—Blood examination showed, haemoglobin 7·4 g., white cells 1200 per c. mm., MCHC 31. Stained films showed moderate anisocytosis of the red cells, but no " tear drop " cells or significant poikilocytosis. There were a number of well-filled macrocytes. Occasional normoblasts were present. No abnormal white cells were noted, the differential count being normal. Platelets were 49,000 pr c. mm. Marrow aspirations were carried out from the sternum and iliac crest. Both yielded very poorly cellular specimens and the scanty marrow elements appeared normal. Myelograms showed erythrocyte/granulocyte ratios of 1 : 2 and 2 : 1 respectively. The findings suggested severe general marrow hypoplasia. A fractional test meal showed free hydrochloric acid. The plasma proteins were 5·4 g. (albumin 3·6 g., globulin 1·8 g.). The alkaline phosphatase was 18 K.A. units.

EXPLANATION OF PLATES

FIG. 1.—Case 1. Aspiration liver biopsy. Multinucleate cells resembling megakaryocytes in various stages of development are present within the sinusoids. There are occasional small groups of myeloid cell precursors. H. and E. × 115.

FIG. 2.—Case 2. Liver biopsy specimen showing focal intrasinusoidal collections of myeloid tissue. The cells are pleomorphic and consist of both red and white cell precursors. H. and E. × 300.

FIG. 3.—Case 3. Lymph node biopsy. There is virtual obliteration of the follicular pattern by a diffuse proliferation of reticulum cells and fibroblast-like cells within the medullary tissues. The impression is of a proliferating cellular fibrosis. H. and E × 50.

FIG. 4.—Case 3. Lymph node biopsy, showing fine and coarse reticulin-staining fibrils separating groups of cells. Reticulin × 360

FIG. 5.—Case 4. Lymph node biopsy. The follicular pattern is inconspicuous. There is a diffuse increase in acellular material, appreciable as a fine fibrillary network involving chiefly the medulla and sinusoids. A very occasional multinucleate cell is present. H. and E. × 40.

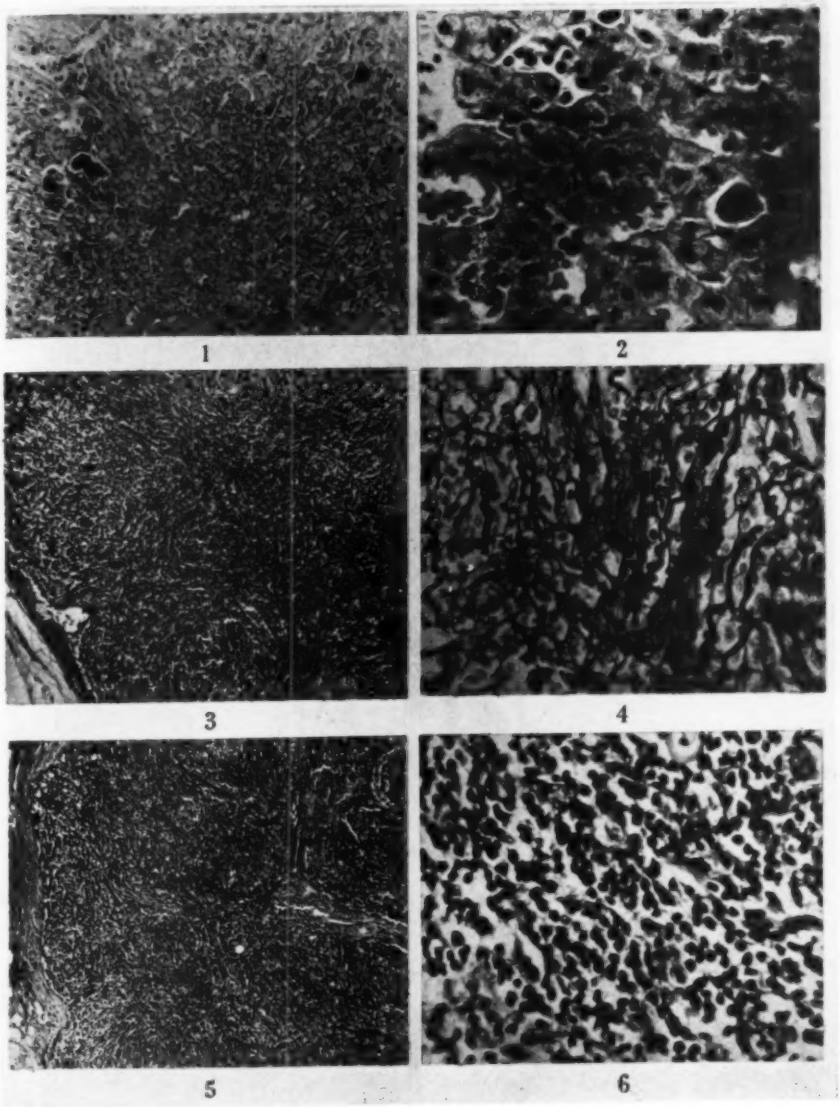
FIG. 6.—Case. 4. Lymph node biopsy. High power appearances of medullary tissues. Fibroblast-like cells are present together with scattered pale-staining reticulum cells. H. and E. × 360.

FIG. 7.—Case 4. Rib biopsy. The marrow architecture is abnormal and typical haemopoietic foci are not apparent. Fibroblast-like cells and small groups of reticulum cells are prominent. There is an increased condensate of fine strands of intercellular fibrillary material. H. and E. × 100.

FIG. 8.—Case 4. Rib biopsy, showing moderately increased amount of reticulin-staining material. Reticulin. × 100.

FIG. 9.—Case 4. Rib (autopsy specimen), showing greatly increased cellularity. The cells are primitive myeloid cells of fairly uniform appearance. Intercellular mesenchymal tissue is inconspicuous. H. and E. × 60.

FIG. 10.—Lymph node (autopsy specimen) from 66-year-old male dying of megakaryocytic myelosis. There is increased cellularity of the sinusoids due to proliferating myeloid tissue. Megakaryocytes are conspicuous. Two lymph follicles are shown, somewhat compressed by the sinusoidal tissues. Involvement of the medullary tissues is less evident, in contrast to the appearances in Fig. 3 and 5. H. and E. × 125.



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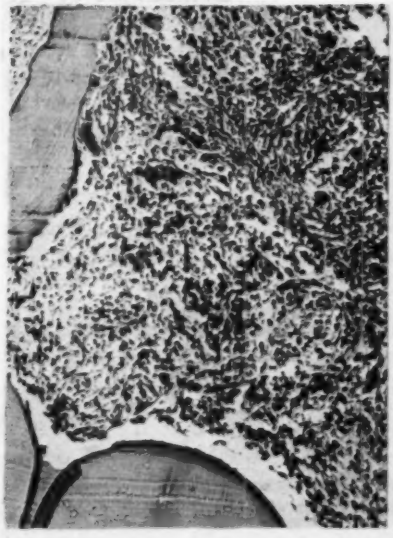

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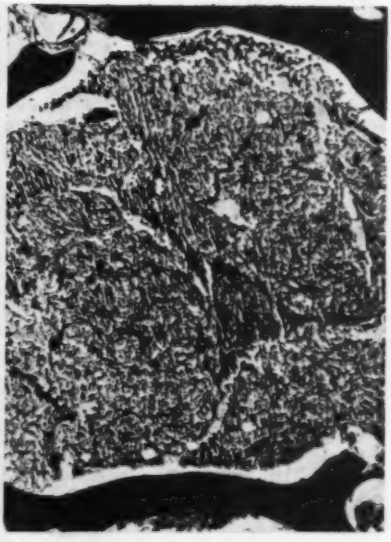

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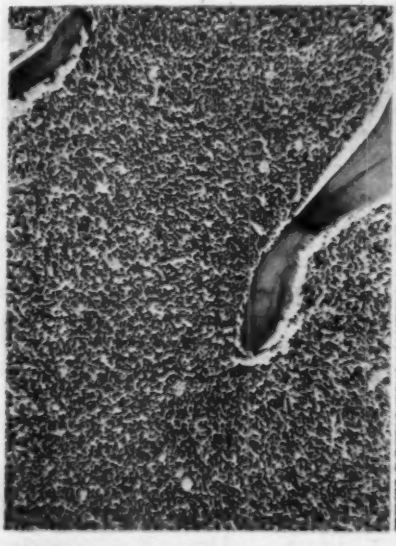

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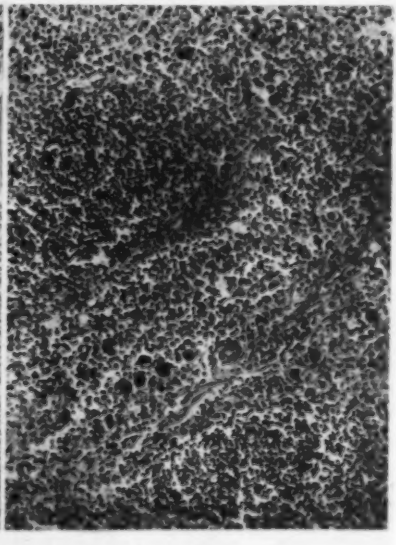

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An inguinal gland was removed. This showed a diffuse loss of normal architecture with atrophy of the lymphoid follicles. The principal components were vascular fibrous tissue and diffuse " reticulum " cell infiltration (Fig. 3). These cells did not appear to be anaplastic. The reticulin pattern was diffusely increased, consisting of numerous long interlacing fibrils (Fig. 4). The appearances were thought to be due to atypical reticulum-cell sarcoma or possibly to myelofibrosis.

A rib biopsy was therefore carried out. This showed a poorly cellular marrow, with loss of the normal architecture, and loss of the normal fat spaces. The diffusely scattered cells present consisted of lymphocytes and fibroblast-like cells and stellate reticulum cells. Only very occasional multinucleate cells were seen. The amount of acellular mesenchymal tissue was greatly increased and stained densely for reticulin. Myelofibrosis was diagnosed.

The patient was transferred to another hospital where she received several blood transfusions. She died at home 4 weeks later. There was no autopsy.

Case 4

A woman aged 57 complained of increasing weakness for 4 weeks, dating the onset from an attack of influenza. On examination she was clinically anaemic ; several discrete rubbery glands were present in the neck, axillae and groins The spleen was slightly enlarged.

Investigation and Progress.—Blood examination revealed haemoglobin 8 g., red cells 2,570,000 per c. mm., white cells 2,700 per c. mm. The stained film showed moderate anisocytosis and poikilocytosis of the red cells. A small number of myelocytes were present. Marrow aspiration (iliac crest) yielded peripheral blood only.

Because of the prominent lymphadenopathy, lymph node biopsy was carried out. Microscopically, there was almost complete loss of normal architecture, with diffuse replacement by cells of a fairly uniform type. No normal lymph follicles remained. The cells resembled reticulum cells with occasional elongated " spindle " cells showing fine protoplasmic processes. The latter resembled fibroblasts. There was a considerable diffuse increase in reticulin staining material (Fig. 5 and 6).

She was given blood transfusions and discharged home for domestic reasons, but an attack of vomiting led to readmission. Again she was very anaemic (haemoglobin 8 g., white cells 2,900 per c. mm.). After further transfusions, rib biopsy was undertaken. The marrow architecture was abnormal. No normal haemopoietic areas were seen. The predominant feature was of strand-like aggregates of mesenchymal cells, closely packed in many areas. The cells resembled stellate reticulum cells and fibroblasts. The intercellular material was much increased and reticulin staining revealed a moderately dense pattern. Multinucleate cells were infrequent. The appearances were those of a moderately cellular myelofibrosis (Fig. 7 and 8).

A course of radiotherapy was given to the glands and spleen. She was discharged, and remained in fairly good general health, though moderately anaemic. She died suddenly 8 months later. No blood examinations had been made in the later stages of illness.

Post-mortem findings.—The state of nutrition was good. The lungs showed general venous congestion and oedema. The myocardium was pale. There was

no significant coronary artery or valvular disease. The liver was enlarged (3240 g.), being pale grey in colour and firm in consistency with appearances suggesting a diffuse infiltration. The spleen (1200 g.) showed no trace of normal architecture and was greyish-pink in colour. There were now no significantly enlarged lymph glands. The marrow of the ribs, sternum and vertebral bodies was uniformly greyish-pink. On microscopy, the heart showed diffuse infiltration by primitive myeloid cells and polymorphs. These were present not only in the distended vessels but in the interstitial tissues. Similar diffuse infiltration was present in the liver sinusoids and involved the connective tissue of the portal tracts and the perivascular connective tissue elements. The liver parenchyma was normal. The splenic tissues showed diffuse replacement by primitive myeloid cells of uniform appearance. Fine strands of reticulin-staining material were present. The kidneys showed diffuse infiltration by similar primitive myeloid cells.

Bone was examined from the ribs and sternum. In both sites the marrow showed an extreme uniform cellularity, the cells belonging to the early myeloid series. Sectioning through the material revealed no areas either of fibrosis or cell pleomorphy. Stellate and fibroblast-like mesenchymal cells were inconspicuous. Reticulin staining strands were observed only very infrequently (Fig. 9).

The post-mortem findings were those of typical myeloid leukaemia.

Comment

Cases 3 and 4 presented with acutely developing anaemia, leucopenia, wasting and lymphadenopathy. The peripheral blood showed moderate anisocytosis of the red cells but " tear drop " cells were not conspicuous, nor were there significant numbers of precursor red and white cells. Marrow aspirates were hypocellular but showed sufficient normal elements to prove unhelpful in suggesting the presence of a myeloproliferative disorder. Attention was directed to the lymphadenopathy.

The histological appearances of the lymph nodes were very similar in both cases. In view of the bone biopsy findings, it was thought possible that a general proliferative process was present in both the lymph nodes and marrow, involving reticulo-endothelial elements and showing partial differentiation towards acellular tissue in both areas.

The abscence of autopsy studies in Case 3 is regrettable. The autopsy findings in Case 4 were those of typical myeloid leukaemia. The findings in the earlier stages of illness in no way suggested this diagnosis. As in Case 2, Case 4 illustrates a possible transitional process from a proliferative process involving lymph nodes and bone marrow, to typical myeloid leukaemia at autopsy.

DISCUSSION

Although primarily disorders of the blood-forming organs, the myeloproliferative syndromes may show extensive extramedullary lesions. Their frequency in autopsied cases of myelofibrosis is illustrated by Vaughan (1936). The value of demonstrating the process in biopsy material is shown in Case 6 of Hutt *et al.* (1953), splenic and liver biopsy supporting a diagnosis of megakaryocytic myelosis, while Korst, Clatanoff and Schilling (1956) in discussing myeloid metaplasia in their cases of myelofibrosis comment that " the incidence (of involvement) might have been higher had spleen and liver biopsies been performed ".

Though lymphadenopathy is only occasionally prominent in the myeloproliferative disorders, histological changes may be more frequent than is usually supposed. The lymph node appearances are well described by Wyatt and Sommers (1950), and Marshall (1956). The usual appearance is of a readily recognisable myeloid tissue infiltrating the sinusoids and often compressing the follicles. Typical megakaryocytes may be conspicuous (Fig. 10). There is a variable proliferation of primitive reticulum cells and fibre-producing cells in the medullary pulp, which may obliterate the normal architecture of the gland. This process was predominant in our Cases 3 and 4.

Assessment of the clinical haematological and histological features will usually allow of differentiation from other processes involving medullary lympho-reticular cell proliferation, such as certain types of reticulum-cell sarcoma, paragranuloma, and histiocytic medullary reticulosis. The possible fallacies in interpreting generalised pathological changes from possibly non-representative biopsy material must be emphasised. In Cases 3 and 4, lymphadenopathy was generalised, and in all the present cases, bone and visceral biopsies were undertaken within a few days of each other. It is tempting to interpret the findings in Cases 3 and 4 as indicating involvement of mesenchymal cells in generalised proliferative disorders affecting various sites of reticulo-endothelial tissue and showing differentiation to acellular tissues.

Increasing use of liver biopsy may reveal the lesions of myeloid metaplasia more frequently in the future. Several types of pathological change may predominate in biopsy material.

(*a*) There may be predominant sinusoidal infiltration by multinucleate cells as in Case 1.

(*b*) Discrete sinusoidal foci of haemopoietic tissue are present in which differentiation to more mature red cell and white cell precursors can be appreciated.

(*c*) There is a diffuse infiltration of the sinusoids by primitive myeloblast-like cells of a relatively uniform type.

In the latter change, the appearances may have to be distinguished from other types of diffuse sinusoidal infiltration. The significance of small numbers of atypical sinusoidal cells in liver biopsy specimens is illustrated by the case of Taylor and Simpson (1950). Serial liver and bone biopsies were successful in revealing the progressive developement of myeloid metaplasia and myelofibrosis. Their recognition is of obvious importance in cases presenting with prominent hepato-splenomegaly.

While adequate blood and bone marrow examinations with bone biopsy are of first importance in diagnosis in the myeloproliferative disorders, and at times in the exclusion of other pathological marrow states, bone biopsy will require experience on the part of the operator and usually a general anaesthetic. There is risk of haematoma and of injuring the pleura in rib biopsy. Lymph node and liver biopsy are established safe and readily repeatable procedures, and appear to be useful ancillary techniques for the diagnosis of these illnesses. The present cases are described for this reason. Their wider use should add to our knowledge of the pathological processes involved, in particular giving better understanding of the natural history and interrelationship of the myeloproliferative disorders and allied conditions.

SUMMARY

(1) The clinical and pathological findings are briefly reported in four patients suffering from myeloproliferative disorders, with termination as myeloid leukaemia in one case. Liver biopsy or lymph node biopsy was carried out in addition to confirmatory bone biopsy at a similar stage of the illness.

(2) Some of the diagnostic problems in these disorders are described; the value of lymph gland and liver biopsy in diagnosis and wider investigation of this group of illnesses is emphasised.

We wish to thank Dr. K. Robertson for kind permission to publish these findings in patients under his care, and Mr. J. Wilcox for the photography.

REFERENCES

HELLER, E. L., LEWISHOHM, M. G. AND PALIN, W. E.—(1947) *Amer. J. Path.*, **23**, 327.
HUTT, M. S. R., PINNIGER, J. L. AND WETHERSLEY-MEIN, G.—(1953) *Blood*, **8**, 295.
KORST, D. R., CLATANOFF, D. V. AND SCHILLING, R. F.—(1956) *Arch. intern. Med.*, **97**, 169.
LEONARD, B. J., ISRÄELS, M. C. G. AND WILKINSON, J. F.—(1957) *Quart. J. Med.*, **26**, 101, 131.
MCMICHAEL, J., and MCNEE, J. W. (1936) *Edinb. med. J.*, **43**, 303.
MARSHALL, A. H. E.—(1956) 'An Outline of the Cytology and Pathology of the Reticular Tissues". First edition, London (Oliver and Boyd), p. 174.
TAYLOR, H. E. AND SIMPSON, W. W.—(1950) *Blood*, **5**, 348.
TUDHOPE, G. R.—(1937) *J. Path. Bact.*, **44**, 99.
VAUGHAN, J.—(1936) *Ibid.*, **42**, 541.
WYATT, J. P. AND SOMMERS, S. C.—(1950) *Blood*, **5**, 329.

STUDIES OF THE EFFECT OF A COLLOIDAL RADIOACTIVE CHROMIC PHOSPHATE ($Cr^{32}PO_4$) IN CLINICAL AND EXPERIMENTAL MALIGNANT EFFUSIONS

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THE work reported here was conducted primarily to assess the behaviour of a colloidal radioactive chromic phosphate, $Cr^{32}PO_4$ injected into serous cavities which contain effusions resulting from neo-plastic processes. This followed a request by the Standing Committee on Radio-Isotopes of the National Health and Medical Research Council (Australia) to investigate the safety and usefulness of the colloidal $Cr^{32}PO_4$ prepared by the Radio-chemical Centre, Amersham, England, since, as far as was known, this particular preparation had not been used clinically before for this purpose. There had been several favourable reports, however, on the use of colloidal $Cr^{32}PO_4$ produced by American laboratories, (Root *et al.*, 1954 ; Jaffe, 1955).

The present studies provide only preliminary results as to the efficacy of colloidal $Cr^{32}PO_4$ in the treatment of malignant effusions. Clinical material was accepted as it became available and as supplies of the colloidal $Cr^{32}PO_4$ were received in this country from Amersham, and careful physical and clinical studies were carried out in each case.

Hitherto in Australia certain malignant serous effusions have been treated in a number of centres with radio-active colloidal gold (^{198}Au) but the quantities used, 100–150 millicuries, create problems in the protection of the staff handling the material and nursing the patient. The substitution of colloidal $Cr^{32}PO_4$ for colloidal ^{198}Au is considered particularly desirable since ^{32}P is a pure beta emitter and the radiation hazard to personnel can be reduced considerably. Furthermore ^{32}P has a longer half life than ^{198}Au and its beta radiation is more energetic and therefore more penetrating. Consideration of these physical properties means that, provided the material is not lost from the cavity, 10 mc. $Cr^{32}PO_4$ will deliver approximately the same amount of radiation to the serous wall as 100 mc. ^{198}Au.

In studies of this nature the fundamental problem always arises as to the rationale of the treatment in question but a considerable ignorance exists regarding the underlying mechanisms of effusions in malignant disease.

The accumulation of fluid in serous cavities may be analysed in terms of a three-compartment model consisting of a vascular bed, serous cavity and lymphatics ; and transfer of fluid normally takes place between these compartments as a dynamic process with the result that in health a minimum of free fluid accumulates in the cavity. Prentice, Siri and Joiner (1952) in studies using water labelled with tritium have shown that a large turnover of peritoneal fluid takes place in patients with ascites. They found that in the presence of 6 litres of ascitic fluid the daily turnover was between 58 and 115 litres. With such a

large turnover only a slight interference with either production or absorption could lead to a large accumulation of fluid.

Straube (1958) in a very carefully planned series of investigations using Ehrlich's ascites tumour in mice has studied the accumulation of fluid during the initial stages of tumour growth, particularly the part played by the presence of malignant cells and possible mechanisms for lymphatic blockage. Whilst neither increase of vascular permeability nor interference with lymphatic drainage and permeability can account entirely for the change observed they can be implicated, particularly the latter.

Adding to confusion are the frequent pathological findings that (1) an intractable effusion in a patient with malignant disease often contains few or no free malignant cells, (2) the effusion may be clear or blood stained, and (3) the associated serosal surface frequently shows minimal signs of malignant ulceration or infiltration. Interpretation of clinical results in such circumstances is extremely difficult and accordingly certain parallel animal experiments were carried out using a controlled malignant ascitic effusion in mice, caused by Ehrlich's transplantable ascites tumour, for which much quantitative information is available. The study of these effusions in mice has the advantage that a direct correlation exists between the accumulation of a protein containing ascitic fluid and the free ascites tumour cell population. It also affords a quantitative study of the earliest collection of this fluid and the effect of agents on the parameters of cellular population and total ascitic volume.

MATERIALS AND METHODS

Radioactive material used

Colloidal $Cr^{32}PO_4$ has been made available from the Radiochemical Centre, Amersham, at monthly intervals. It is a colloidal suspension prepared by grinding ignited chromic phosphate in water. The product is centrifuged to remove large particles and is dialysed to remove ionic material before despatch. No stabilizing agent is used. The particle size is given as approximately 0·05 μ with aggregates up to 0·3 μ. (Charlton, 1958, personal communication).

The first consignment received was one of 5 mc., thereafter consignments were of the order of 12 mc. It was not always possible to use the material as soon as it arrived because suitable patients were not always available, and in addition to loss by decay doses were further reduced by the aliquots taken for various physical studies. The whole of the remainder of the consignment of $Cr^{32}PO_4$ was administered to the patient in each case and the doses ranged from 4·8 mc. to 12·9 mc.

Clinical material

Ideally the natural history of the individual's disease should be relatively benign, the history (particularly of the serous effusion) should be long and there should be no severe symptoms which are not referable to the presence of the fluid. Cases at a terminal stage with malnutrition and anaemia are unsuitable, and another contra-indication in the case of ascites is the presence of palpable masses in the abdomen and any other evidence of the probability of gross adhesions causing loculation. This selection of patients has been easier to describe than to perform and because the colloidal $Cr^{32}PO_4$ has been received only at regular

intervals it has not been possible on all occasions to insist on the above clinical indications.

This series of cases included 9 with pleural effusion and 7 with peritoneal effusion caused by a variety of primary tumours as detailed in Table I.

CLINICAL METHOD

On receipt of each consignment of $Cr^{32}PO_4$ an aliquot of the solution was diluted in distilled water and dialysed for 24 hours using Nojax casing (a cellulose casing containing glycerine, water and a little sulphur) which has a permeability

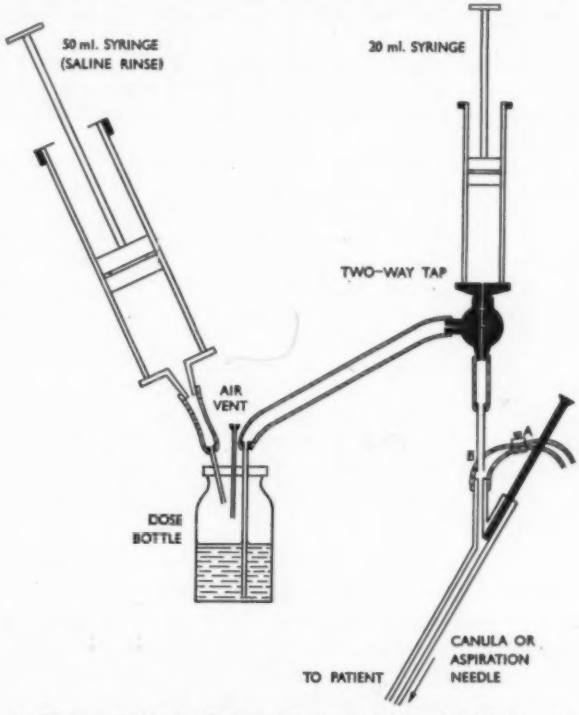

FIG. 1.—Technique for the intracavity administration of $Cr^{32}PO_4$ (see text).

of approximately 2·4 mμ. The solution was then autoclaved ready for administration to the patient. In the last case reported here (J. B—) the whole dose was dialysed for 24 hours and the quantity remaining in the sac after this period was then autoclaved for administration. Occasionally the solution flocculated but while this was undesirable it was not considered a contra-indication to its use. It could be resuspended by shaking after which it settled out slowly.

Administration was carried out as follows (Fig. 1). Sufficient fluid was withdrawn from the serous cavity so that as far as could be foreseen no further paracentesis would be necessary for at least five or six days; this allowed time for

the $Cr^{32}PO_4$ administered into the cavity to plate out on to the serous wall. It was the practice not to attempt to drain the cavity but to leave 2 or 3 pints to ensure that the $Cr^{32}PO_4$ was introduced into a reasonable volume thereby improving the likelihood of a good distribution throughout the cavity.

Following paracentesis the rubber tubing of the canula side arm was clamped off at A and pierced by the needle B of the 20 ml. syringe assembly. $Cr^{32}PO_4$ was withdrawn from the dose bottle into the 20 ml. syringe and introduced into the serous cavity via needle B and the canula. Several saline rinses from the 50 ml. syringe were introduced into the dose bottle, withdrawn into the 20 ml. syringe and introduced into the serous cavity in a similar manner to the dose. The whole apparatus was thus well rinsed and the dose was given in a total volume of about 70 ml.

In order to obtain as good a distribution of $Cr^{32}PO_4$ as possible the patient was required to turn a quarter of a turn every quarter of an hour for the subsequent two hours, the foot of the bed being raised for the first hour and the head of the bed for the second hour.

Using a collimated scintillation counter, containing a NaI (Tl) crystal, to detect the ^{32}P bremsstrahlung a radio-active survey of the patient was carried out about three hours after administration in order to ascertain how well the ^{32}P was distributed throughout the serous cavity, and a similar survey was made after a few days to check the distribution.

Samples of serous fluid and blood were taken for radioactive assay at two hours after administration and at other times during the first six or seven days. Urine was collected in 24 hour samples for several days for radioactive assay.

Peripheral blood cell counts were carried out where possible for several weeks.

Two cases (M.G., a pleural effusion and F.O'D., a peritoneal effusion) came to autopsy. Samples of pleura and its underlying muscle, peritoneum and diaphragm were taken and the surface areas measured. These samples were then digested in 10 N Nitric Acid and assayed using an M6 liquid counter.

Quantities of ^{32}P have been corrected in all cases to the time of administration of the corresponding dose.

EXPERIMENTAL METHODS

Hybrid Mice of the Walter and Eliza Hall Institute stock weighing 25–30 g. were used throughout in these experiments. The transplantable Ehrlich ascites tumour used was the hyperdiploid line ELD (Lettrè) with a 46 chromosome mode. The tumour was passaged and maintained in C3H mice in the laboratories of the Peter MacCallum Clinic.

For a given experiment tumour cells from a single donor mouse were used to inoculate both control and treated animals. The donor cell population for inoculation was aspirated and counted in a Neubauer haemocytometer after dilution in Tyrode solution at 37·5° C. Before counting the sample was further diluted in a 0·05 per cent solution of eosin (Gurr; Water Soluble Yellowish) in phosphate buffered saline pH 7·4 (Dulbecco and Vogt, 1954), and a differential count of viable and non-viable tumour cells was made according to the method of Hoskins, Meynell and Sanders (1956). Usually an inoculation dose of 2×10^6 tumour cells was used per mouse and given intraperitoneally. The mice were weighed daily throughout each experiment and the incidence of lethality recorded.

To observe the effect of various size doses of $Cr^{32}PO_4$ mice were given single intraperitoneal injections varying from 20 μc. to 80 μc. per mouse at 48 hours after inoculation of the animals with the tumour (Groups II–V). This range of doses was chosen because an intraperitoneal dose of 20 μc. $Cr^{32}PO_4$ in mouse was considered approximately equivalent to an intraperitoneal dose of 10 mc. $Cr^{32}PO_4$ in man, based on estimates of the volume of the peritoneal cavity in the two cases.

Subsequently the effect of fractionation of $Cr^{32}PO_4$ was studied in mice using two intraperitoneal injections of 75 μc. at 3 and 7 days after inoculation.

In separate groups of control and treated mice, inoculated with tumour cells, small samples of ascitic fluid were aspirated at chosen times after inoculation and differential counts made of viable and non-viable cells. Random samples were also examined for tumour cell morphology using Leishman's stain, and for metaphase chromosome abnormalities using Diller's orcein squash technique in hypotonic solution. The abdomens of mice which died were opened, the ascitic fluid measured and the distribution and extent of solid tumour deposits were recorded.

RESULTS

Clinical progress of patients

The clinical results in the 16 cases in this study are detailed in Table I. Clinical relief was marked in 8 patients (7 with pleural and 1 with peritoneal effusion); some improvement was observed in 3 patients (1 with pleural and 2 with peritoneal effusion); and no improvement was observed in the other 5 patients (1 with pleural and 4 with peritoneal effusion.)

In the five cases in which there was no improvement, two had palpable abdominal masses and the other three showed rapid clinical deterioration.

Malignant cells were demonstrated in 13 cases before treatment (8 with pleural and 5 with peritoneal effusion) and were not found in the remaining 3 cases (1 with pleural and 2 with peritoneal effusion).

No significant blood changes attributable to the treatment were observed.

Dialysis of $Cr^{32}PO_4$

The results of dialysis tests on 14 consignments of $Cr^{32}PO_4$ are given in Table II. The percentage amount of ^{32}P which dialysed in 24 hours varied considerably from 4–23 per cent, but most of the results lie between 10–20 per cent. There is no correlation between the percentage dialysed and the time interval between despatch and dialysis.

Distribution of ^{32}P in the serous cavity measured by external counter

Although the measurement of bremsstrahlung from the ^{32}P in the serous cavity is not regarded as quantitative it does indicate the gross distribution of the ^{32}P. Fig. 2 shows a good distribution of $Cr^{32}PO_4$ in a peritoneal cavity (Patient E. V—) and Fig. 3 shows a poor distribution in a peritoneal cavity a few hours after administration in a patient (M. S—) with abdominal adhesions. In this latter case the $Cr^{32}PO_4$ showed only moderate dispersion over four days at which time the second survey was made (Fig. 4). It is interesting to note that in this case the specific radioactivity of the peritoneal fluid fell from 10·5 μc./ml. to 0·1 μc./ml. during this time.

TABLE I.—*Clinical Observations in 16 Patients with Malignant Serous Effusions Treated with* $Cr^{32}PO_4$

Name	Age	Sex	Diagnosis	Cytology	Specific previous treatment	Dosage of $Cr^{32}PO_4$ (mc.)	Distribution of $Cr^{32}PO_4$ in serous cavity	Clinical relief	Survival since onset of symptoms as at 31.xii.58	Survival since injection of $Cr^{32}PO_4$ as at 31.xii.58
E. O'K—	46	M.	Pleural effusion Ca. lung	Malignant cells present	*Nil*	5·2	Good	Marked	15 wks.	3 wks. Died.
M. G—	71	F.	Ditto	Ditto	X-ray therapy to thorax	12·0	,,	,,	5½ mths.	2½ mths. Died.
J. C—	45	M.	Pleural effusion. Reticulum cell sarcoma	,,	Ditto	4·8	,,	Some	17 mths.	11 mths. Died.
C. J—	43	F.	Pleural effusion. Malignant melanoma	,,	*Nil*	11·2	,,	None	12 mths.	2½ mths. Died.
M. F—	63	M.	Pleural effusion. Ca. bronchus	,,	X-ray therapy to thorax	9·5	,,	Marked	11 mths.	4 mths. Died.
L. S—	66	F.	Pleural effusion. Ca. breast	No malignant cells found	Oestrogens	6·6	,,	,,	11 yrs.	5 mths.
J. S—	66	M.	Pleural effusion. Primary unknown	Malignant cells present	*Nil*	12·9	,,	,,	1 yr.	3½ mths.
J. L—	65	,,	Ditto	Ditto	,,	9·7	,,	,,	18 mths.	2½ mths.
J. B—	70	F.	Pleural effusion. Ca. Breast	,,	Oestrogens	10·5	,,	,,	7 mths.	1½ mths.
M. O'D—	54	,,	Ascites. Ca. ovary*	No malignant cells found	*Nil*	7·0	Loculated	None	Over 2 yrs.	5½ mths. Died.
F. O'D—	59	,,	Ascites. Ca. breast*	Malignant cells present	Oestrogens	8·2	,,	,,	Over 6 mths.	12 days. Died.
E. V—	57	M.	Ascites. Ca. bile duct	No malignant cells found	Surgical relief of obstructive jaundice	11·0	Good	Marked	2 yrs.	10 mths. Died.
O. W—	61	F.	Ascites Ca. ovary	Malignant cells present	*Nil*	9·0	,,	Some	5 mths.	4½ mths. Died.
M. P—	57	,,	Ditto	Ditto	Oöphorectomy. X-ray therapy	4·6	,,	None	3 yrs.	6 wks. Died.
C. S—	67	,,	,,	,,	*Nil*	6·3	,,	,,	3 mths.	2½ mths. Died.
M. S—	46	,,	,,	,,	Nitramin	12·3	Loculated	Some	3 mths.	1 mth.

* Abdominal masses present.

Three hours after administration when the first survey is carried out most of the $Cr^{32}PO_4$ is still in the serous fluid, but by the time the second survey is made much of it has plated out on to the walls of the serous cavity. Although the $Cr^{32}PO_4$ is differently distributed on these two occasions a comparison of the two surveys may be made, and in each case it has indicated that most of the ^{32}P has been retained in the cavity.

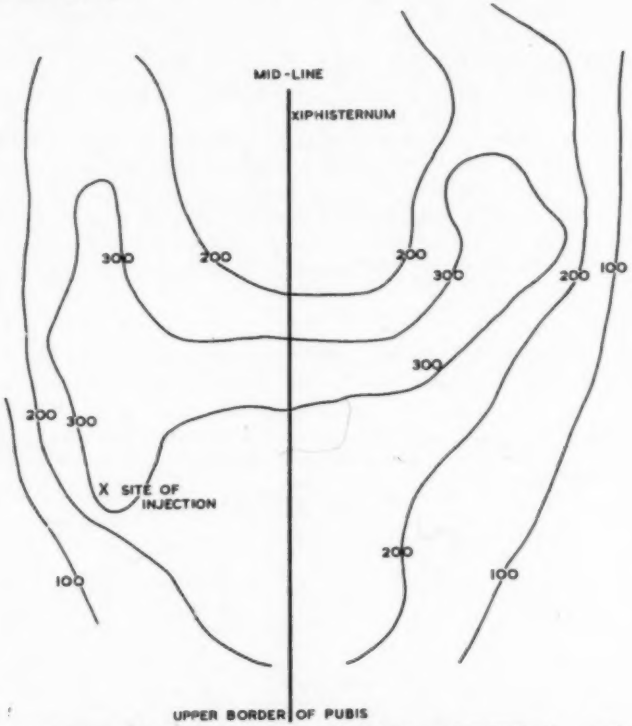

FIG. 2.—A radioactive survey showing a good distribution of $Cr^{32}PO_4$ in a peritoneal cavity (Patient E. V—). Numbers represent relative counting rates.

Urinary excretion of ^{32}P

The ^{32}P contained in the urine of patients given $Cr^{32}PO_4$ has been measured for four days after administration, and the percentage of the dose excreted in each case is given in Table II. For all the patients except J. B—, the dose was not dialysed prior to administration and it was found that between 1·5–5 per cent of the dose was excreted, the average being approximately 2·5 per cent. For patient J. B—, the dose was dialysed prior to administration and the amount excreted was thereby reduced to 0·6 per cent. Dialysis of the urine showed that all of the ^{32}P was in ionic form.

In order to calculate from these excretion figures what percentage of the $Cr^{32}PO_4$ dose reached the blood in ionic form, three patients were given a known

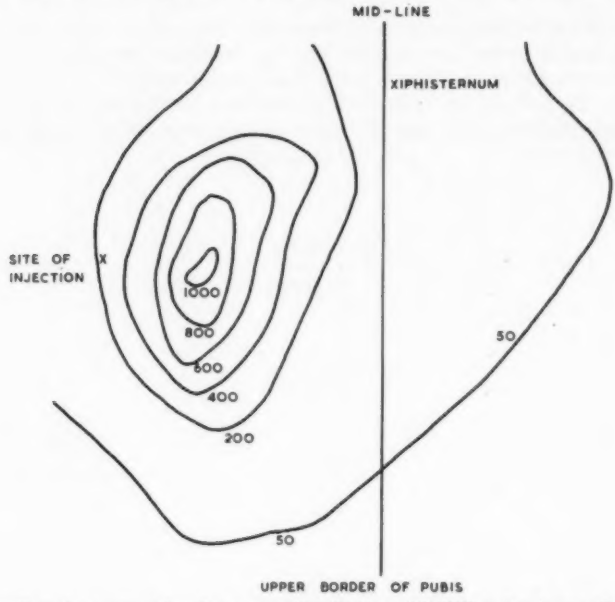

FIG. 3.—A radioactive survey showing a poor distribution of $Cr^{32}PO_4$ a few hours after administration, in a patient (M. S—) with abdominal adhesions. Numbers represent relative counting rates.

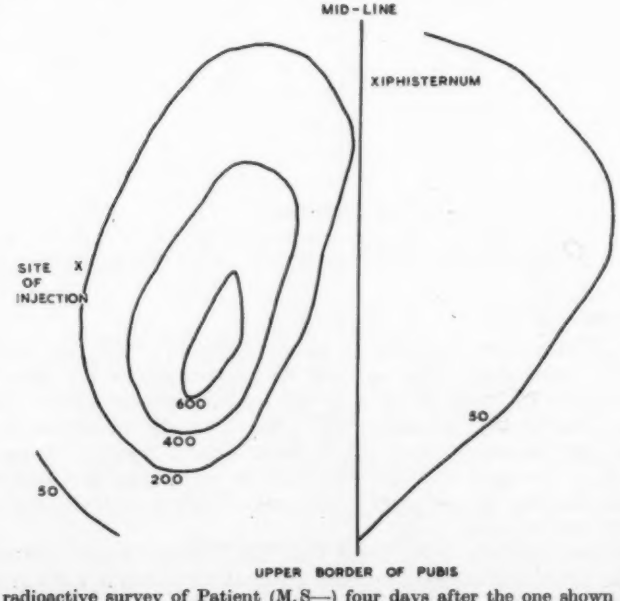

FIG. 4.—A radioactive survey of Patient (M.S—) four days after the one shown in Fig. 3, showing that the dispersion of the $Cr^{32}PO_4$ during this period is limited. Numbers represent relative counting rates.

intravenous dose of $Na_2H^{32}PO_4$ and the amount of ^{32}P excreted in the urine in four days was measured. The results were 13, 16 and 17 per cent with an average of approximately 15 per cent.

Assuming that in both the $Cr^{32}PO_4$ and $Na_2H^{32}PO_4$ groups of patients the ^{32}P excreted in 4 days is related to the amount of ionic radiophosphate reaching the blood then :—

$$\frac{\text{Excretion of } ^{32}P \text{ following } Na_2H^{32}PO_4}{\text{Dose of } Na_2H^{32}PO_4 \text{ reaching blood } (= 100\%)} = \frac{\text{Excretion of } ^{32}P \text{ following } Cr^{32}PO_4}{\text{Amount of } ^{32}P \text{ from the } Cr^{32}PO_4 \text{ dose reaching blood.}}$$

Comparison of these excretion data indicates that when the $Cr^{32}PO_4$ is not dialysed prior to administration then approximately 16 per cent of the ^{32}P contained in the dose reaches the blood in ionic form in a very short time. This figure agrees very well with the average of 15 per cent of the dose found to dialyse.

When the $Cr^{32}PO_4$ is dialysed prior to administration the excretion figures indicate that only about 4 per cent of the ^{32}P reaches the blood.

TABLE II.—*Results of Dialysis of $Cr^{32}PO_4$, and the ^{32}P content of Serous Fluid, Urine and Blood following the intracavitary Administration of $Cr^{32}PO_4$*

Patient	Percentage of $Cr^{32}PO_4$ dialysing in 24 hours (%)	Amount of $Cr^{32}PO_4$ administered (mc.)	Concentration of ^{32}P in serous fluid in μc./ml.10 mc. dose at 2 hours	3–5 days	Later	Percentage of dose excreted in urine in 4 days	Amount of ^{32}P in blood During 1st week (% of dose)	Amount of ^{32}P in blood Later (% of dose)
Pleural effusions—								
E. O'K—	—	5·2	7·8	0·3	0·1 at day 29	—	1	1 at day 13
M. G—	—	12·0	6·0	0·4	<0·1 „ „ 14	2·1	1·5–2	1 „ „ 14
E. C—	—	4·8	3·7	0·3	—	5·0	1	—
E. J—	—	11·2	14·2	0·2	—	2·3	1–1·5	—
M. F—	12	9·5	7·7	2·4	1·8 „ „ 7	2·3	1	1 „ „ 7
E. S—	15	6·6	33·6	8·9	—	2·7	1–1·5	—
E. S—	12	12·9	19·1	8·4	6·4 „ „ 6	2·6	0·5–1	0·5 „ „ 26
E. L—	—	9·7	3·5	1·0	—	3·7	1·5	—
E. B—*	13	10·5	3·2	0·1	—	0·6*	0·2*	—
Peritoneal effusions—								
M. O'D—	—	7·0	3·0 (loculated)	—	—	2·2	2·5–3·5	—
E. O'D—	10	8·2	4·2	0·1	0·1 „ „ 11	2·0	1·5	—
E. V—	4	11·0	3·6	0·1	0·1 „ „ 6	3·0	1	0·7 „ „ 6
D. W—	12	9·0	1·3	0·6	—	1·6	1·5–2·5	—
M. P—	12	4·6	2·9	0·3	0·2 „ „ 6	2·0	1	—
E. S—	21	6·3	1·2	<0·1	—	2·2	1·5	—
M. S—	16	12·3	10·5 (loculated)	<0·1	0·06 „ „ 8	3·7	1–1·5	—
Other consignments of $Cr^{32}PO_4$—								
	18	—	—	—	—	—	—	—
	23	—	—	—	—	—	—	—
	20	—	—	—	—	—	—	—
	14	—	—	—	—	—	—	—

* Whole dose dialysed prior to administration.

Specific concentration of ^{32}P in serous fluid

The specific concentrations of ^{32}P in the serous fluid at 2 hours, 3–5 days and at longer intervals after administration of the $Cr^{32}PO_4$ are given in Table II in terms of $\mu c./ml./10$ mc. dose. In most cases the specific radioactivity fell to less than 10 per cent by the fourth day, a change which is much too great to be explained by any increase in volume of the serous fluid.

In four patients (M. F—, O. W—, L. S—, J. S—) the fall in specific radioactivity was much less marked and the reason for this variation is not understood.

The specific radioactivity of the serous fluid was generally much lower for peritoneal effusions than for pleural effusions.

^{32}P in blood

The amount of ^{32}P circulating in the blood remained reasonably constant over the usual period of study of one week and showed only a slight fall in later samples taken as long as 26 days after administration of the $Cr^{32}PO_4$. The results are given in Table II. In patient J. B—, where dialysis of the $Cr^{32}PO_4$ dose was carried out prior to administration the concentration of ^{32}P in the blood was appreciably reduced.

The proportion of the circulating ^{32}P incorporated in the red cells builds up to 70–85 per cent over the first few hours after intracavity administration of $Cr^{32}PO_4$, after which it remained fairly constant for as long as measurements were made. The same pattern was followed after intravenous injection of $Na_2H^{32}PO_4$.

Distribution of $Cr^{32}PO_4$. At autopsy

The radioactive concentration ($Cr^{32}PO_4$) of various samples of pleura or peritoneum from the two cases which came to autopsy are given in Table III in terms of $\mu c./sq.cm.$ per 10 mc. dose.

The striking feature of these results is the very wide variation of the order of 100 : 1. In case M. G—, it could be argued that the $Cr^{32}PO_4$ may have settled

TABLE III.—*Distribution of $Cr^{32}PO_4$ over the Serous Wall in Two Cases at Autopsy*

(Corrected to the time of administration)

Patient M. G— Died 2½ months after $Cr^{32}PO_4$ Pleural effusion	$\mu c./sq. cm.$ per 10 mc. dose	Patient F. O'D— Died 12 days after $Cr^{32}PO_4$ Peritoneal effusion	$\mu c./sq. cm.$ per 10 mc. dose
Pleura, upper lobe	0·13	Left post peritoneum	0·38
Pleura (tumour) upper lobe	0·12	Right post peritoneum	0·09
Parietal pleura (superior)	0·17	Centre post peritoneum	0·06
Parietal pleura (tumour) (inferior)	0·43	Right lat. peritoneum	1·1
Parietal pleura (inferior)	2·3	Left ant. peritoneum	0·2
Diaphragm	17·0	Right ant. peritoneum	0·5
		Left diaphragm peritoneum	0·03
		Right diaphragm	1·4
		Peritoneum over spleen	0·1
		Wall of transverse colon	3·6

under gravity to the lower regions of the pleural cavity, but there is no apparent pattern in the results of Case F. O'D—.

In Case M. G—, assay of the muscle underlying the pleura indicated a very marked fall off in radioactivity within a millimetre of the pleural surface.

Results on experimental animals

Survival studies.—All inoculated animals in both control and treated groups died. The mean survival for various groups is recorded in Table IV.

Single injections of $Cr^{32}PO_4$ in doses from 20 μc.–80 μc. per mouse failed to influence significantly the lethality from Ehrlich ascites tumour. When the dose was repeated (Group IX) and 75 μc. given on the third and seventh days of tumour

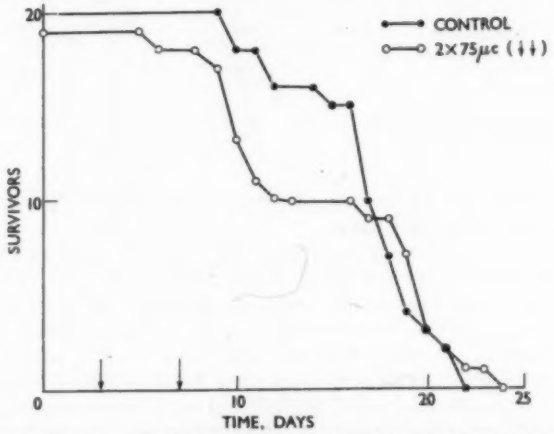

FIG. 5.—Survival curves for control inoculated mice injected with saline and inoculated mice receiving 2 doses of 75 μc. $Cr^{32}PO_4$ intraperitoneally, on 3rd and 7th day after inoculation, respectively (see text).

growth, the survival curve showed a biphasic trend, and nearly 50 per cent of animals died rapidly. apparently the result of the effects of total body irradiation (Fig. 5).

Accumulation of Ascites Fluid.—Colloidal $Cr^{32}PO_4$ in doses of 20–80 μc. given on the second day after inoculation caused a decrease in the usual gain in animal weight recorded over the first twelve days after inoculation with the tumour (Table IV, Groups II–V). A similar striking result was seen after the administration of two doses of 75 μc. on the third and seventh days of tumour growth (Group X).

This alteration in weight increase was due to a diminution in the accumulation of ascitic fluid. Measurements of daily fluid intake of treated and control mice revealed no essential difference in the daily consumption. For treated animal groups the mean daily fluid consumption per mouse for the first seven days after injection of $Cr^{32}PO_4$ ranged from 4·5 ml. to 6·9 ml., whilst control animals gave a corresponding range of 4·6 to 5·9 ml. This finding differs from that observed by van den Brenk and Parsons (1958) in the treatment of tumour inoculated mice with orally administered hydrogen peroxide, where the reduction in ascitic

TABLE IV.—*Effect of Colloidal $Cr^{32}PO_4$ Administered Intraperitoneally to Hybrid Walter and Eliza Hall Mice at Various Times after Inoculation with 2×10^6 cells of Ehrlich ascites Tumour*

Group	Number of animals	Mean animal weight on day of inoculation (g.)	Mean survival time (± SD) (Days)	Weight increase or loss over first 12 days after inoculation (g.)
I. Control (for Groups II–V)	9	42·8	16·6 (±2·1)	+8·5
II. 20 μc. at 48 hours	9	46·0	15·8 (±1·9)	+1·1
III. 40 μc. at 48 hours	10	43·1	17·6 (±3·1)	+0·1
IV. 60 μc. at 48 hours	10	46·5	17·4 (±3·0)	−1·0
V. 80 μc. at 48 hours	10	41·5	19·0 (±6·2)	−3·8
VI. Controls (for Groups VII and VIII)	10	28·1	18·6 (±1·3)	—
VII. 20 μc. at 24 hours	10	28·5	18·0 (±1·5)	—
VIII. 20 μc. at 8 days	10	29·0	16·6 (±2·5)	—
IX. Control (for Group X)	20	36·6	17·0 (±3·6)	+13·1
X. 2 × 75 μc. at 3 and 7 days	19	37·1	15·3 (±5·6)	+7·5

distension and body weight corresponded to reduced fluid intake and general dehydration of the treated animals.

Post-mortem examinations of $Cr^{32}PO_4$ treated mice showed a marked reduction of ascitic fluid, but large masses of tumour were adherent to mesenteries and abdominal viscera. The measurement of residual ascitic fluid *post-mortem* was an unsatisfactory determination owing to the interval which elapsed between death and the examination. Similarly the measurement of the ascitic fluid volume *in vivo* using dye dilution techniques was very unsatisfactory owing to turbidity of the fluids, the high contamination of the fluid with blood cells in many animals, and loculation of fluid in the treated mice in particular.

Tumour cell population.—Repeated measurements of the cell population in separate groups of control and treated animals showed a relative reduction in the number of tumour cells per unit volume ascitic fluid in the $Cr^{32}PO_4$ treated animals (Fig. 6). This reduction was particularly marked on the fourth day after administration of the $Cr^{32}PO_4$. Thereafter the tumour cell counts in all groups continued to fall, approaching a similar low level which was maintained until death of the animals.

Differential counts of non-viable cells, using the eosin technique, were made and in the control animals a sharp rise in the non-viable count occurred in the days immediately preceding death of the animals. The non-viable count was not raised in the isotope treated animals nor did it show the sharp rise before death recorded in control mice; it would appear that the effect of the irradiation is to inhibit cellular proliferation.

DISCUSSION

In most of the 16 cases studied the fall in the specific radioactivity of the serous fluid is far too great to be explained by an increase in its volume, a feature which has been observed by others (Root *et al.*, 1954), and since the body surveys indicate that most of the ^{32}P remains in the serous cavity it must be concluded

that the $Cr^{32}PO_4$ plates out on to the serous wall. The rate at which this plating out process takes place varies considerably but in most cases it is nearly complete after a few days. The concentration of this ^{32}P over the serous wall however, has been shown from samples taken at two autopsies to vary very widely by a factor of 100 and determination of the dose of $Cr^{32}PO_4$ to be administered must therefore be empirical.

In this series no difference in dose has been made to allow for the smaller area of pleural cavities compared with peritoneal cavities, as suggested by Lange, Shields and Rozendaal (1956), or to allow for the various types of primary tumour. The size of the doses used for patients (Table I) shows no relation to the clinical results which followed. On the other hand, in the animal experiments where

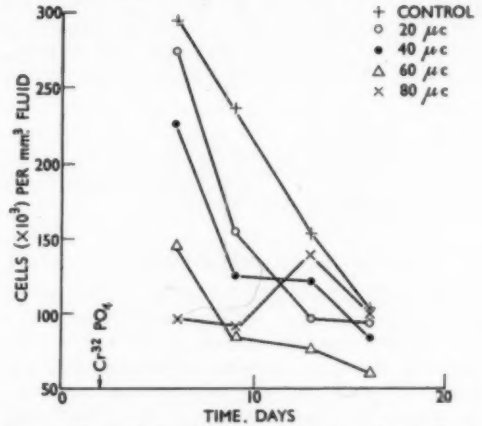

FIG. 6.—Progressive tumour cell counts in ascitic fluid at various times after inoculation of mice with 2×10^6 cells Ehrlich ascites tumour, in control animals and animals treated with single intraperitoneal injections of $Cr^{32}PO_4$ 48 hours after inoculation.

conditions were better controlled a definite relationship was observed between dose and both fluid formation and cell counts.

Instability of a $Cr^{32}PO_4$ preparation not only reduces its efficacy as a source of local radiation of a serous cavity but may produce an unacceptable amount of ^{32}P in ionic form with rapid access to bone marrow.

On receipt of the $Cr^{32}PO_4$ about 15 per cent of the ^{32}P is found to be in ionic form and, as the material is dialysed prior to despatch from Amersham, this quantity must dissociate during the flight of approximately five days between England and Australia. The doses of $Cr^{32}PO_4$ used have been of the order of 10 mc. so the amount of ionic radioactive phosphate present amounts to about $1\frac{1}{2}$ mc. This is an undesirable feature and one that can be largely removed by dialysis of the material just prior to administration, but if facilities are not available for dialysis the amount of ionic radiophosphate present is not considered unacceptable for this type of case. To what extent further ionization of the $Cr^{32}PO_4$ continues after absorption of the colloid by the serous wall is not known, but the hazard of additional radioactive phosphate being absorbed is considered to be very small.

In animal experiments, not reported here, the effects of ^{198}Au and Nitramin on fluid formation and cell counts have been shown to be similar to those observed using $Cr^{32}PO_4$. This is consistent with reports of clinical experience with these agents (e.g. Hilton *et al.* 1957; Bonte, Storaasli and Weisberger, 1956) and the 8 out of 16 patients in the present series who showed marked clinical improvement following $Cr^{32}PO_4$ is a comparable result.

CONCLUSION AND SUMMARY

It is considered that the use of radioactive colloidal chromic phosphate, as prepared by the Radiochemical Centre, Amersham, can be recommended for the treatment of malignant serous effusions since the hazard to the patient is small, particularly if the material is dialysed prior to administration. There are no toxic effects as experienced with cytotoxic drugs, and the hazard to staff handling the material and nursing the patient is negligible compared with radioactive colloidal gold.

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REFERENCES

Bonte, F. J., Storaasli, J. P., and Weisberger, A. S.—(1956) *Radiology*, **67**, 63.
van den Brenk, H. A. S., and Parsons, I. C.—(1958) *Med. J. Aust.*, **1**, 633.
Dulbecco, R., and Vogt, M.—(1954) *J. exp. Med.*, **99**, 167.
Hilton, G., Halnan, K. E., Howard, N., and Godfrey, B. E.—(1957) *J. Fac. Radiol., Lond.*, **8**, 339.
Hoskins, J. M., Meynell, G. G., and Sanders, F. K.—(1956) *Exp. Cell. Res.*, **11**, 297.
Jaffe, H. L.—(1955) *Amer. J. Roentgenol.*, **74**, 657.
Lange, R. H., Shields, J. L., and Rozendaal, H. M.—(1956) *N.Y. St. J. Med.*, **56**, 1928.
Prentice, T. C., Siri, W., and Joiner, E. E.—(1952) *Amer. J. Med.*, **13**, 668.
Root, S. W., Tyor, M. P., Andrews, G. A., and Kniseley, R. M.—(1954) *Radiology*, **63**, 251.
Straube, R. L.—(1958) *Cancer Res.*, **18**, 57.

THE COMPOSITION OF CIGARETTE SMOKE: STUDIES ON STUBS AND TIPS

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In earlier publications studies were made of the amounts of a number of polycyclic aromatic hydrocarbons in mainstream cigarette smoke (Cooper and Lindsey, 1955) and in the residual stubs and ash (Gilbert and Lindsey, 1956). These quantities showed that, after allowing for the amounts present in the unsmoked material, (Campbell and Lindsey, 1956) considerable quantities were trapped in the stubs and very small amounts were found in the ash. From these and other studies a mechanism for the smoking process was proposed (Gilbert and Lindsey, 1956) elaborating previous suggestions such as those of Wenusch (1939). In the present paper, studies of the mainstream smoke with varying stub length give additional support to these proposals and are also shown to be in harmony with statistical studies in U.S.A. (Hammond, 1958) and in Great Britain (Doll, Hill, Gray and Parr, 1959) on the relationship between lung cancer mortality and stub length.

The amounts of condensible compounds in mainstream cigarette smoke vary as the process goes on and are smaller at the commencement and heavier towards the end of a cigarette. It is therefore important to examine the composition of the inspired smoke at various stages during the smoking process and to relate this with the proposed mechanism of smoke formation.

Outline of the smoking process

The glowing zone of a cigarette during intervals between puffs is at about 650° C. and varies very little in temperature throughout its volume. During the suction periods the temperature rises by about 50° C. although superficial temperatures may be well over 900° C. It has also been shown that the temperature gradient behind the glowing zone is very steep; about 600° in 4 mm. (Harlow, 1956). During the quiescent or smouldering process, in a short region behind the glowing zone, distillation of various volatile constituents including water occurs and the products condense a little further back. In the region nearer the glowing zone thermal decomposition of the relatively non-volatile components occurs and some of the products of this process distil into the cooler regions while another portion emerges from the glowing zone in the side-stream smoke. In particular, amides and proteins give rise at this stage to ammonia and this is mainly responsible for the alkaline nature of the side stream smoke although the mainstream smoke may be acidic because of the excess of acidic constituents in it.

During suction, combustion is rapid and the distillation and thermal decomposition processes also occur much more quickly. The products, hot gases and steam from the combustion with extra steam from volatilized water, effect rapid steam distillation from the adjacent zones and the " smoke ", an aerosol consisting

of droplets of a few microns in diameter, is quickly drawn through. Some of the aerosol condenses throughout the whole length of the cigarette. The repetitive distillation of products into a cool region near the glowing zone during quiescent periods and the more rapid distillation and steam distillation during suction produces a certain amount of fractionation so that the condensed material at later stages in the smoking process is richer in less volatile materials than at earlier stages. Thus, during later stages, there is more thermal decomposition of distilled materials with higher boiling points than occurs in earlier stages.

This outline of the smoking process although based upon existing knowledge is in part conjectural. It is now additionally supported by the following studies.

EXPERIMENTAL

Determination of rate of production of condensible material in main-stream smoke

Cigarettes with the desired stub length marked on the paper were smoked mechanically one by one in an apparatus similar to, but smaller than, that used

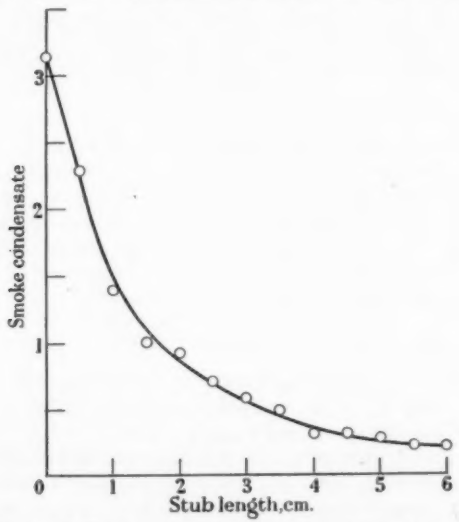

FIG. 1.—Condensible matter in mainstream cigarette smoke measured for various stub lengths. Amount from 1·5 cm. stub = 1.

previously (Cooper and Lindsey, 1955). The smoke was condensed in acetone which absorbed most of it; the rest was allowed to settle and then the whole was transferred quantitatively with additional solvent into a graduated flask. The total amount of condensible matter obtained upon smoking a cigarette to a definite stub length was found by examining an aliquot portion of the acetone solution spectrophotometrically. The whole experiment was repeated several times and the average quantities are plotted in Fig. 1, whence it is seen that in the smoke from short stubs much greater quantities of condensible matter are present than from long stubs.

Examination of Mainstream Smoke and of Stubs for Polycyclic Aromatic Hydrocarbons

The glass apparatus was cleaned and all solvents and reagents were purified as described previously. Smoking experiments were carried out with the machine formerly used and the conditions of smoking were as then described. Stub lengths of 1·5 and 3·5 cm. were chosen, the former as the result of a survey of the habits of 150 British smokers (Commins, Cooper and Lindsey, 1954), and the latter because this represents half consumption of the cigarette of normal size (7·0 cm.).

The mainstream smoke was analysed by alumina chromatography and ultra-violet spectrophotometry as previously described and the stubs, after drying over sulphuric acid were extracted with cyclohexane in a Soxhlet extractor and the hydrocarbons determined in the neutral fraction in the same way.

The results are shown in Table I as micrograms of hydrocarbon from 100 cigarettes or 100 stubs.

TABLE I.—*Polycyclic Aromatic Hydrocarbons in Cigarette Smoke and in Stubs*

Hydrocarbon	Stub length 1·5 cm.		Stub length 3·5 cm.	
	Smoke	Stubs	Smoke	Stubs
Anthracene	22	12 (4)	23	42
Pyrene	15	5 (0·6)	7	17
Fluoranthene	10	36 (6)	8	12
1 : 2-Benzanthracene	14	9 (6)	8	14
3 : 4-Benzpyrene	3	2 (0·9)	0·7	3

The amounts shown in parenthesis were obtained by analysis of stubs collected by Doll and his colleagues in their statistical studies.

The lower molecular weight and more volatile hydrocarbons are held in greater proportion in the long stubs than in the shorter stubs. It also appears that the lighter molecular weight compounds are more subject to destruction during the smoking process.

Examination of Tipped Cigarettes

A great variety of tips and filters for cigarettes are in use. Selective removal of smoke constituents is claimed for some whereas less precise claims are made for others. Bearing in mind the nature of mainstream cigarette smoke, especially the small particle diameters and the high velocity of the aerosol during inspiration, it might be anticipated that smoke trapped in various tips or filters would be of the same composition as that of the smoke passing through them. Preliminary experiments have shown this to be so (Cooper and Lindsey, 1955).

Accordingly the smoke trapped in the tips of a type popularly in use in Great Britain were analysed. They consist of a roll 1·5 cm. long of crimped paper tightly compressed within a paper cover fitted within an outer cover to the cigarette. Each paper filling weighed 0·2 g. and provided a total absorbing area of 156 square cm. These cigarettes were of the same tobacco as those used in previous experiments, and were smoked mechanically to about 1 cm. from the commencement of the tip. The unsmoked tobacco was cut from the tips and the latter after drying over sulphuric acid were extracted to exhaustion with cyclohexane. The

quantities of polycyclic aromatic hydrocarbons found in the extract are shown in Table II.

TABLE II.—*Polycyclic Aromatic Hydrocarbons in tips from* 100 *Cigarettes in Micrograms*

Anthracene	0·4 (4·6)
Pyrene	1·3 (2·5)
Fluoranthene . . .	1·0 (1·6)
1 : 2-Benzanthracene . .	0·6 —
3 : 4-Benzpyrene . . .	0·3 (4·4)

The amounts shown in parenthesis were obtained by analysis of tips collected by Doll and his colleagues in their statistical studies.

The stubs used by Doll and his colleagues (1959) in their measurements of length were recently analysed for polycyclic aromatic hydrocarbons by an improved technique (Lindsey, 1959). All the tipped stubs were segregated from the collection and separately examined. They were mostly (all but 2) of the same kind of rolled paper as those smoked mechanically and described above. A number (146) of the remaining stubs were randomly selected and analysed after drying. The results are shown in Tables I and II in brackets for ease of comparison with the results of mechanical smoking.

DISCUSSION

The determination of total condensible mainstream smoke issuing from cigarettes at progressive stages in the smoking process reveals that there is a great deal of condensation of smoke in the tobacco during smoking and that, due to redistillation processes, the early smoke drawn contains much less condensible material than the later smoke.

Examination of the total smoke drawn when long stubs (3·5 cm.) are left shows that the amounts of higher molecular weight hydrocarbons are much less than when short stubs (1·5 cm.) are discarded. Thus the smoke from 100 cigarettes leaving long stubs contains less than one quarter of the 3 : 4-benzpyrene found in the smoke from the same number smoked to the short length. If this compound or any other known carcinogen (or as yet unknown carcinogen) in smoke is a factor in lung cancer causation, then it is likely to have less effect in populations smoking to longer stub lengths.

The results from the tips and stubs obtained from the survey of Doll and his colleagues do not agree closely with those obtained by mechanical smoking. There may be a number of reasons for this. In connection with the tips it was found that those collected from smokers were smoked to extremely short tobacco stubs, indeed some had no tobacco left on them. The average weight of tobacco left by the smokers was 0·065 g. whereas the tipped cigarettes were mechanically smoked to leave 0·214 g. This could well account for the higher figures shown in brackets in Table II.

Other causes of the differences could be the great variation in stub length, the variation in original tobacco and the long interval (over six months) between smoking and analysis of the stubs provided by Doll and his colleagues.

SUMMARY

Studies have been made of the total amounts of condensible smoke produced at various stages in the smoking of cigarettes and it has been demonstrated that the stubs retain large quantities of such condensible matter. The amounts are in harmony with the idea of a succession of distillation processes during the smoking sequence.

The smoke from cigarettes smoked to long and short stubs was analysed for polycyclic aromatic hydrocarbons and it was shown that from the same number of cigarettes four times as much 3 : 4-benzpyrene was found in the smoke when short stubs were left as when long ones were discarded. This is in harmony with statistical studies of lung cancer mortality and stub length in smoking populations. Smoking to a short stub has less effect on the amounts of other hydrocarbons.

Tips were also studied and it was shown that they retain far less polycyclic aromatic hydrocarbons than stubs of similar length.

The author wishes to thank the Medical Research Council for supporting these investigations by the loan of apparatus and provision of technical assistance and materials. He also thanks Dr. Richard Doll, O.B.E., for providing the stubs used in the statistical survey of stub length in Great Britian.

REFERENCES

Campbell, J. M. and Lindsey, A. J.—(1956) *Brit. J. Cancer*, **10**, 649.
Commins, B. T., Cooper, R. L. and Lindsey, A. J.—(1954) *Ibid.*, **8**, 296.
Cooper, R. L. and Lindsey, A. J.—(1955) *Ibid.*, **9**, 304.
Doll, R., Hill, A. B., Gray, P. G. and Parr, E. A.—(1959) *Brit. med. J.*, **1**, 322.
Gilbert, J. A. S. and Lindsey, A. J.—(1956) *Brit. J. Cancer*, **10**, 642.
Harlow, E. S.—(1956) *Science*, **123**, 226.
Hammond, E. C.—(1958) *Brit. med. J.*, **2**, 649.
Lindsey, A. J.—(1959) *Analyt. chim. acta*, **20**, 175.
Wenusch, A.—(1939) ' Der Tabakrauch '. Bremen (Arthur Geist Verlag), p. 18.

ACTIVE CELLULAR LYSIS, A PHENOMENON OF GROWTH PROCESSES AND ITS ROLE IN THE FORMATION OF DIFFERENT EPITHELIAL PATTERNS AS SHOWN IN MAMMARY CARCINOMAS IN MICE*

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It is generally assumed that the histologic pattern which the growth of a particular tumor takes is in some way determined by an intrinsic characteristic of the tumor cell. But the great influence exerted by the host tissue or the medium in which the tumor is growing in determination of its histologic pattern as well as its biological behavior, has not been denied.

In the course of the microscopical study of a large number of mammary carcinomata of mice, it was realized that the developmental processes of tumors depend not only on cellular multiplication but also on lysis (dissolution) of the tumor tissue, including both parenchyma and stroma, in varied patterns. It appears that cell dissolution is as important as cellular multiplication in formation and maintenance of the structural patterns, and the present study deals with the developmental processes of a tumor growing in its natural host.

MATERIALS AND METHOD

The observations presented are based on the study of over 1500 mammary carcinomata in C3H female mice. This includes 1048 transplanted and 71 spontaneous tumors obtained at autopsy examination; a part of the same material was used in a previous paper (Ghosh, 1959). It also includes biopsy specimens obtained from 44 spontaneous tumors and specimens obtained by antemortem excision of 315 transplanted carcinomas and 51 spontaneous carcinomas. A large number of the tumour-bearing animals were used in our experimental chemotherapy program. No appreciable difference was noticed between treated and untreated groups, with respect to the histologic picture of the tumor.

Tumors were fixed in 10 per cent neutral formalin and sectioned at 4 micron thickness and stained with hematoxylin and eosin for routine study. For confirmation of the shapes of certain histological structures in their entirety in three dimensions, serial sections of the tumors made at 4 to 6 micron thickness were examined. Special stains for the detection of mucin, amyloid, iron, calcium, hemoglobin, melanin, elastic tissue, reticulin, muscle, fat, PAS positive material, etc. were employed on some specimens. Both light and phase contrast microscopy were used.

* A partial presentation of this paper was made at the Fifty-fifth Annual Meeting of the American Association of Pathologists and Bacteriologists.

OBSERVATIONS

The formation of various histological patterns in carcinoma was studied by observing the cellular changes which developed during the growth of mammary carcinomata in mice. In this paper the observations are limited to the various cellular changes associated with cell lysis and to reorientation of the surviving and newly formed cells into several histologic designs with retention of their epithelial nature.

In the course of the lytic process, which involves both epithelial and stromal elements, various intermediate stages are recognisable, which may be called digestion, degeneration and necrosis. After first noting each of the cellular changes leading to dissolution and the changing characters of the fluid, we shall illustrate the lytic phenomena, as well as the cellular growth and arrangement in the formation of various tumor patterns. By digestion, we mean simply the dissolving out of the cytoplasmic and nuclear substances, as one sees in the case of cells digested *in vitro* by various proteolytic enzymes, without any evidence of degeneration or necrosis. Cellular lysis has been seen associated with changes which fall under the category of various degenerative processes, such as hyaline, hydropic, fibrinoid, granular, vacuolar (watery or fatty), mucoid, myxoid, amyloid, keratoid, etc. The granular and vacuolar configurations have been termed the secretory stage and the lytic product, when observed within a lumen and bound by a lining epithelium has been called secretion. Cellular lysis may be preceded by necrosis. During this process, in certain stages the cellular changes may be indistinguishable from what is usually considered as pus in inflammatory conditions. Sometimes a cellular change known as aseptic necrosis may precede cell lysis. No vascular obstruction is noticed to explain the necrosis. Necrosis was seen in tumor nodules measuring less than 1 mm. in diameter. One or more than one of the cellular changes associated with lysis mentioned above may take place in a group of cells.

The product of cellular lysis (secretion) may exhibit further and various changes in its biochemical make-up, as reflected by the varied nature of its staining characteristics from one area to another: clear (vacuoles), various shades of colors, granulation, globule formation, rarely calcification and others. The changing characters of the fluid have often been termed maturation. By using different staining techniques, reactions in the fluid become even more prominent. In this paper we shall not deal with these phenomena in any detail, since only black and white prints of photomicrographs are presented and since its purpose is to draw attention to the phenomenon of cellular lysis as an active process in the growth of a tumor.

Since the normal mammary gland presents a tubo-acinar pattern primarily, the developmental processes of an acinar structure in tumors are presented first (Fig. 1–6). The formation of the acinar lumen and accumulation of fluid in it, as a result of cellular lysis involving scattered round areas, can be well followed in each of Fig. 2, 3 and 6. Here one can also notice the stages of reorientation of the surviving peripheral cells in the formation of cuboidal epithelium, which encircles the products of cell lysis. In what appears to be a quiescent stage of developmental processes including both cellular lysis and multiplication (a rapid rate of which is shown in Fig. 7) and when in contact with fluid, the inner margin of the surviving cells forming lining epithelium becomes rounded and a well differentiated single cell layer epithelium is formed.

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In the process of the formation of acinar fluid from a solid cell mass, one may notice clumping of cells with hyalinization (Fig. 2) or vacuolation of the cells in the acinar lumina (Fig. 2 and 3). In Fig. 3 one can see the fading away of the cells with the appearance of watery vacuoles in the interstices of the remnants of lysing cells and formation of homogeneous coagulable fluid. Here stages of " maturation " of the fluid and organization of the lining epithelium are going on hand-in-hand toward formation of well differentiated acini. The formation of tubo acinar structures from solid cell masses in cancer was recognized by early workers (Murray, 1908 ; Gierke, 1908) and the origin of tubo acinar structures of the mammary gland from solid cell masses was observed by both histologists and embryologists (Maximow and Bloom, 1957 ; Arey, 1940 ; Bailey and Miller, 1921 ; McMurrich, 1920). They pointed out that the appearance of the lumina in these structures is caused by a separation and breaking down of the central cells of ducts and acini, the peripheral cells remaining as their lining.

In Fig. 5 and 6, which simulate lactating mammary glands, one can see cellular lysis associated with fatty vacuolation of the cytoplasm and nucleus toward formation of acinar fluid. The lysis of cells following fatty changes (secretion, degeneration) can be seen in the process of differentiation toward acini. Some cells showing fatty vacuoles can be seen in the inter-acinar areas. Foulds (1956) has pointed out the prominent milky secretion in spontaneous and transplanted mammary carcinomas of the mouse when the animals are pregnant, lactating, or receive estrogens. He also stated, " Occasionally it is questionable whether the accumulation of fat should be ascribed to secretion or degeneration."

In an area of a well differentiated epithelial pattern (acinar or other types) with a single layer of lining cells with more or less rounded margin, the rates of cellular lysis and formation of new replacement cells appear to be slow and balance each other.

It is possible that when the lytic process in the acinar cells is slow compared to cellular multiplication, the acinar pattern is gradually obliterated. In such acini, concentric layers or crowding of lining cells may be found because of the persistence of inner layer cells due to delay in lysis, as shown in Fig. 4. The enlargement of the lumen (often called dilatation) of a glandular structure appears to be due to the peripheral extension of the area of cellular lysis and to the new epithelium forming at an outer zone, instead of replacing the previous one *in situ*.

In some areas a high rate of cellular multiplication can be seen in the acinar and inter-acinar cells, associated with rapid cellular lysis, where there is hardly any cytoplasmic differentiation (Fig. 7). It is conceivable that with a relative slowing down of the speed of cellular lysis, this can be transformed into a solid sheet of poorly differentiated cells, as shown in Fig. 8, in which crowded nuclei can be seen lying in a pool of cytoplasm. On close examination, many scattered nuclei can be seen in various stages of lysis. This stage may be followed by a more quiescent stage, where the speed of cellular multiplication is slow. In such areas (Fig. 9) one may notice the attempts at differentiation into a glandular pattern from a solid sheet of tumor cells. Both Murray (1908) and Gierke (1908) have pointed this out.

The lytic processes sometimes lead to the formation of scattered small cysts, as seen in the formation of the adenoid cystic pattern (Fig. 10). Myxoid, mucoid, hyaline, hydropic or fibrinoid changes are usually associated with cellular lysis in the origin of the adenoid cystic pattern. The surviving lining cells lack radial

arrangement, unlike what is seen in the differentiation of the acini ; flat, spindle or " oat " cells may form the lining of the cysts.

By studying serial sections, observations have been made on the continuity of the lumina of many acini or cystic structures among themselves, with the formation of channels (tubes, ducts). The formation of channels by dissolution of the contiguous walls of the acini has been noticed. When such acini are arranged in columns, regular tubes may be formed with unification of their lumina (Fig. 11, 12). In what appears to be the earlier stages of tubal formation from acini, one can see a corrugated appearance of tubes because the acini forming the tubes have not yet completely lost their previous pattern. Tubes may also be formed by the lysis of a column of centrally placed cells either in a continuous process, or by the lysis of cells in separate groups at various levels of the cell column before joining each other (Fig. 14). The development of basket cells of so-called " myo-epithelial " nature from the peripheral epithelial cells which touch the connective tissue, as described by Maximow and Bloom (1957), can be seen in this figure. The formation of a narrow tube by dissolution of a central cell layer has also been observed (Fig. 13).

The lytic process can sometimes be seen involving large areas of the tumour at a stretch, often preceded by necrosis. The stages from early necrotic change to the formation of fluid giving rise to cystic cavities can be followed in Fig. 15–18. Even at an early stage, a sharp line of demarcation may be seen between the sheet of cells destined to lyse and the peripheral surviving cells which form the lining cells of the cystic cavity (Fig. 15). Such large cyst formation may naturally develop from a variety of pre-existing tumor patterns with various amounts of stromal elements. Necrotic tissue in some stages of liquefaction may resemble pus (Fig. 16) of inflammatory origin. In Fig. 16 and 17, one can see the stages of dissolution of necrotic tissue and formation of fluid in the cystic cavities and, at the same time, proliferation of the surviving lining cells to form polypoid projections into the cysts. A similar histologic structure has been known as intra-cystic papilloma or intraductal papilloma. On serial sections, communications between the cystic cavities forming channels are commonly observed. One may sometimes notice the formation of secondary cysts through the lysis of the central substances of the polypoid mass inside a mother cyst (Fig. 17).

The origin of large cystic spaces from cellular lysis, preceded by keratinization, can occasionally be seen. This is more common in certain tumor lines than others. Keratinization may or may not be preceded by squamous cell transformation.

Often the lysis of tumor cells may follow a papilliferous or papillary cystic anastomosing pattern (Fig. 19–30). With the process of lysis going on in the lumen, the surviving cells at the periphery attempt to organize themselves into columnar or cuboidal epithelium lining the papillae. The lining of these papillae bordering the fluid becomes rounded off when the cells constituting the lining epithelium cease to lyse and their inner surface is in contact with fluid in the lumen. When there is delay in separation of a sheet of lysing cells from the surviving peripheral cells, there appears to be a delay or imperfection in assuming the pattern of columnar or cuboidal epithelium; then a tendency towards formation of spindle or flat cells from surviving lining cells can be seen, as shown in Fig. 23.

The cleft-like spaces between the two opposite sides of the lining epithelium may vary from very narrow sinuses to large cystic spaces, as shown in Fig. 19–30. Here the formation of the fluid as a result of cellular lysis associated with different

EXPLANATION OF PLATES

The photomicrographs presented here are made from 5 spontaneous and 9 transplanted mammary carcinomas in C3H female mice ; none of these mice were pregnant or lactating during the period they had tumors. All transplanted carcinomas are of second generation, except that shown in Fig. 11 which is a third generation tumor. All these tumors were obtained at fresh autopsy examination, except the tumor in Fig. 13 and 14, which is an ante-mortem specimen.

The photomicrographs presented here are made from sections stained with hematoxylin and eosin.

FIG. 1 and 2.—These two photomicrographs, taken from the same tumor, present an acinous pattern. In Fig. 2 at higher magnification, remnants of liquefying cells are seen in the acinar lumen. Fig. 1. × 145. Fig. 2. × 500.

FIG. 3.—Stages of developmental processes in the formation of a well differentiated acinous structure from a solid cell mass. × 540.

FIG. 4.—Crowding of cells with formation of irregular concentric layers in the lining epithelium and obliteration of some acinar lumina by cells showing varying degrees of liquefaction. × 415.

FIG. 5 and 6.—These two photomicrographs, taken from the same tumor, demonstrate vacuolar (fatty) changes in the tumor cell mass and various stages of origin of acinar structures from these cells. Vacuolation can be seen in the acinar fluid, in lining cells of the acini, as well as in the solid cellular areas. Acinar differentiation can be better followed in Fig. 6. Fig. 5. × 215. Fig. 6. × 395.

FIG. 7.—This photomicrograph was taken from an area from which the acinar pattern is almost obliterated by the rapid rates of cellular lysis and cell multiplication. There is hardly any cytoplasmic differentiation of the cells. × 990.

FIG. 8.—Crowding of the nuclei and loss of epithelial differentiation, with formation of a sheet of undifferentiated cells. × 395.

FIG. 9.—The epithelial nature of the cells is slightly more revealed here, even in the solid area in the upper part of the photomicrograph, compared to the preceding photomicrograph. There is a sign of differentiation to glandular pattern among the solid mass. × 495.

FIG. 10.—Cellular lysis resulting in small scattered cystic spaces is presented in this area, forming what is known as the adenoid cystic pattern. Note the lack of radial arrangement of the lining cells as shown in acinar differentiation. × 260.

FIG. 11 and 12. The formation of tubal structures from acini is shown here. There is lysis of the adjacent cell layers of acini arranged in columns. Fig. 11. × 470. Fig. 12. × 500.

FIG. 13.—Origin of a narrow tubular structure from lysis of the centrally placed cell layer is shown here. × 650.

FIG. 14.—Formation of a large tube or duct from a solid cell mass by cellular lysis. Note the differentiation of the basal layer cells into basket cells (' myoepithelial ') type. × 270.

FIG. 15, 16, 17.—These photomicrographs taken from the same tumor show the different stages of formation of cysts or irregular channels (often termed as dilated ducts) and intracystic polypoid structures. Note the liquefying processes involving the central areas of the polypoid structures (Fig. 17) toward formation of cysts within cysts. Fig. 15. × 595. Fig. 16. × 80. Fig. 17. × 100.

FIG. 18.—This photomicrograph demonstrates varying sized cysts or irregular channels formed by lysis of the tissue. Remnants of unliquefied tumor tissue can be seen in the lumina of the cysts. × 195.

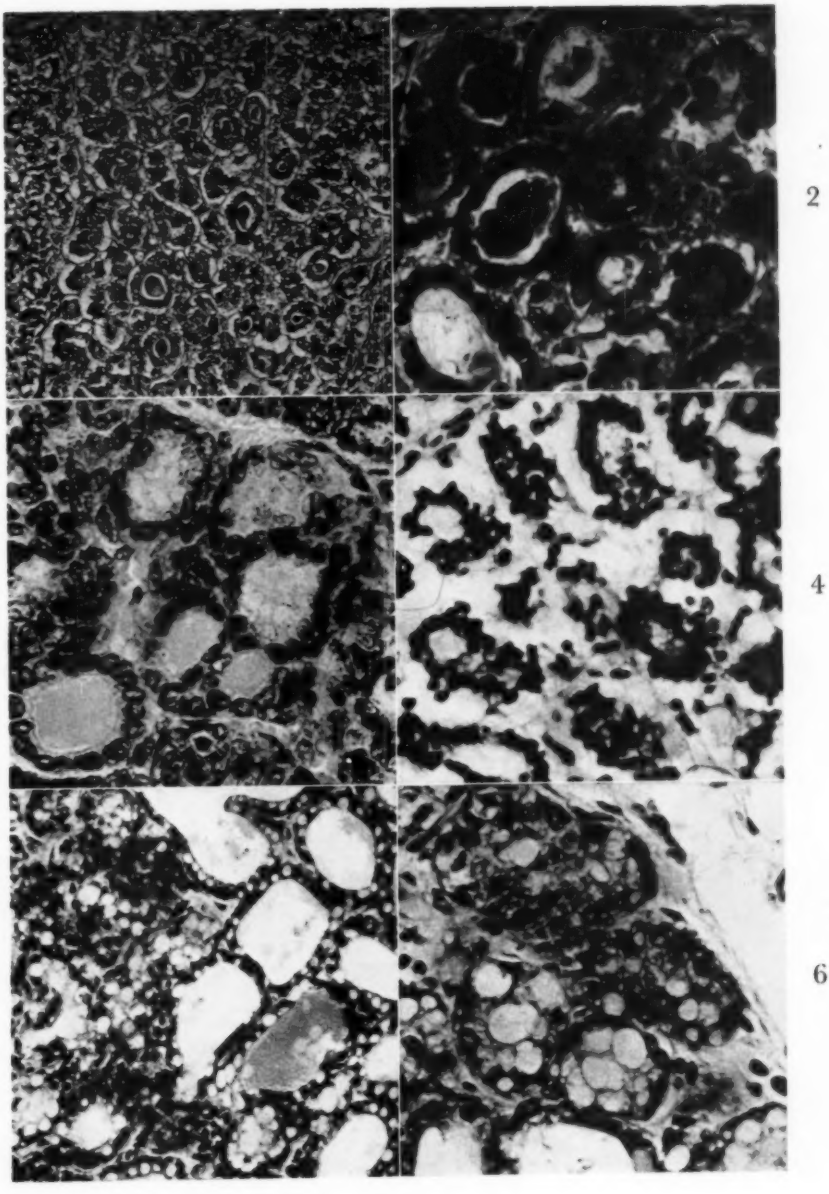
FIG. 19, 20 and 21 are taken from the same tumor.

FIG. 19.—A papilliferous carcinoma pattern is shown here at low magnification. × 85.

FIG. 20.—This is a high power view of the upper central area of the preceding figure. This shows cellular digestion leading to the formation of slit-like spaces between the surviving tumor cells ; the latter form the lining epithelium. A high rate of cellular multiplication with crowding of the cells can also be noticed in the epithelium. × 540.

FIG. 21.—The formation of a slit-like space preceded by necrosis is shown in the center. Note the peripheral surviving cells orienting themselves into columnar epithelium as they are being detached from the necrotic cells. Cellular digestion in formation of narrow slit-like spaces can be seen in other areas. In the left lower part of the field one can see a tiny portion of well differentiated cuboidal epithelium, the margin of which is rounded off. × 500.

FIG. 22.—Prominent necrosis of the tumor tissue and emergence of a papillary cystic pattern are demonstrated. Note the vacuolar change in the tumor cells lying on the course of separation between the surviving cells lining the papillae and the neighboring necrosed tissue. Compare this with Fig. 26, where vacuolar change of the cells is more prominent than necrosis preceding cell liquefaction. × 125.



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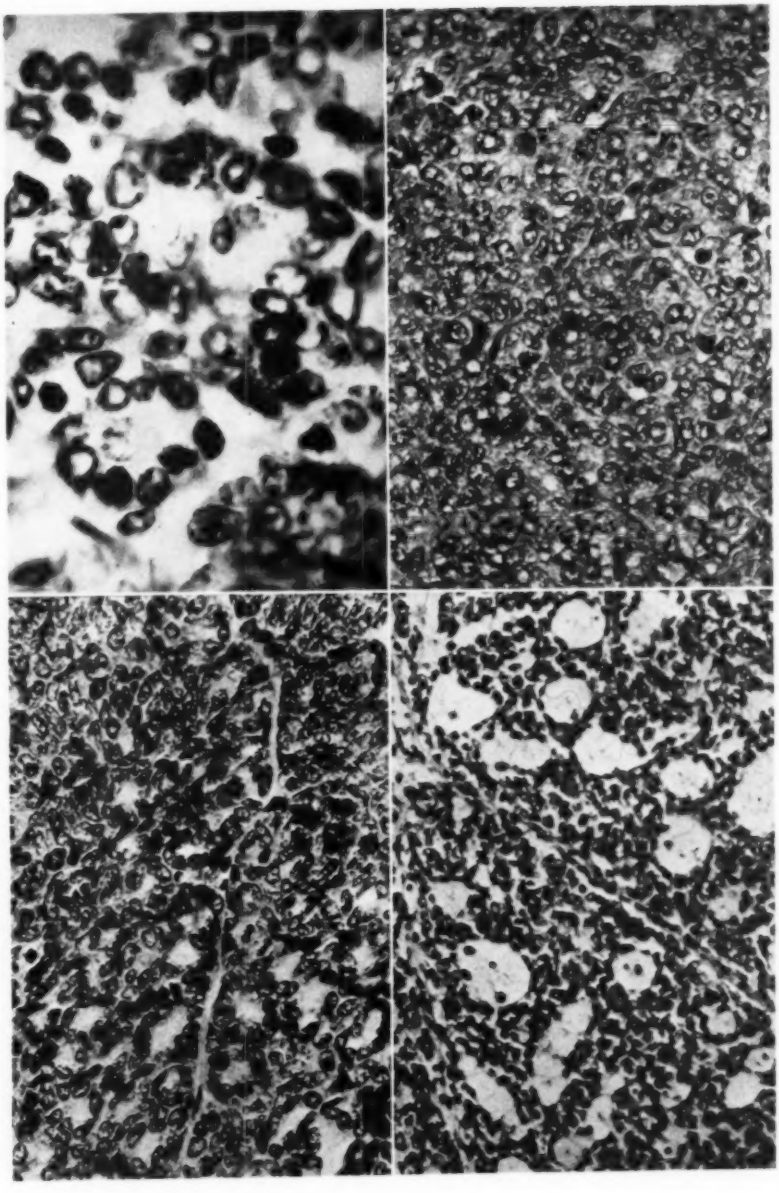

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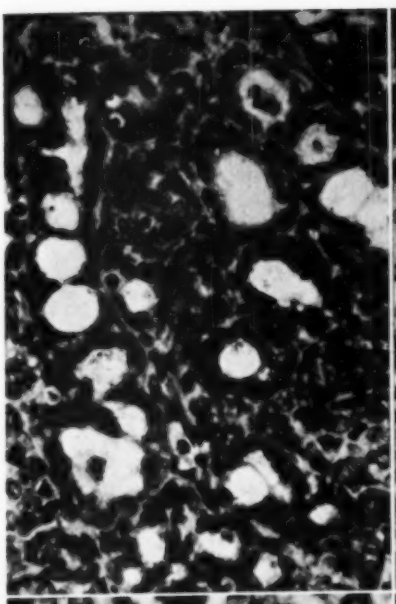

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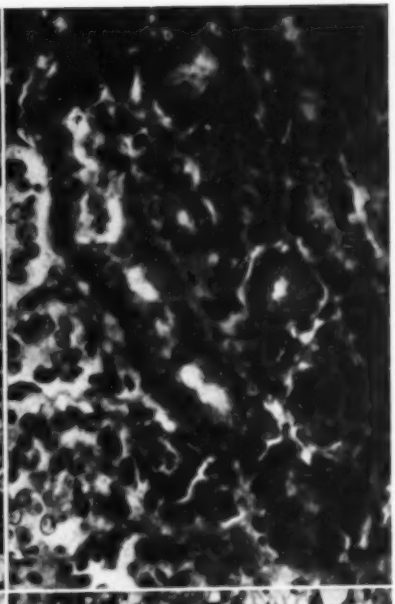

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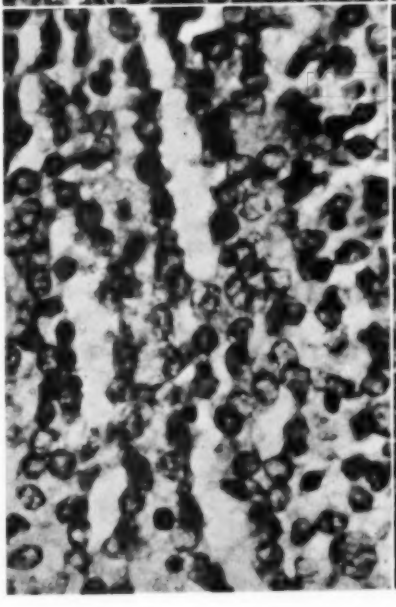

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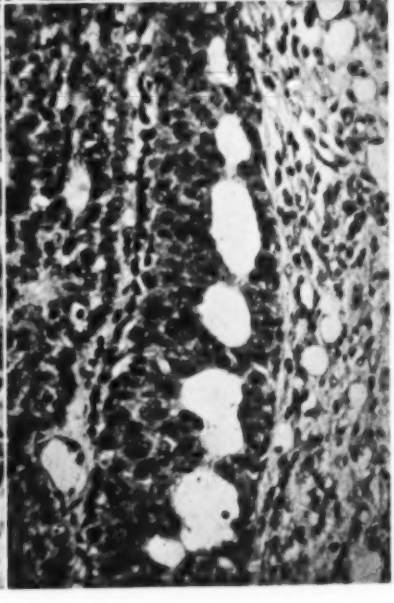

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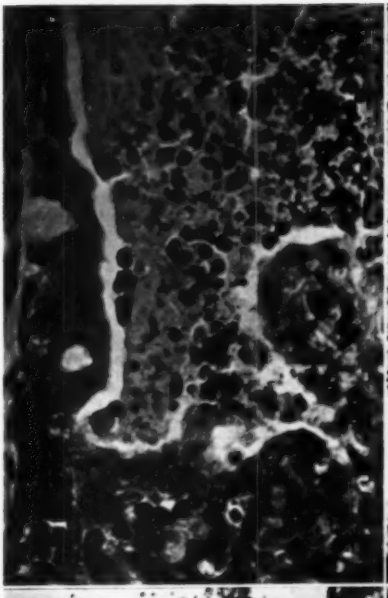

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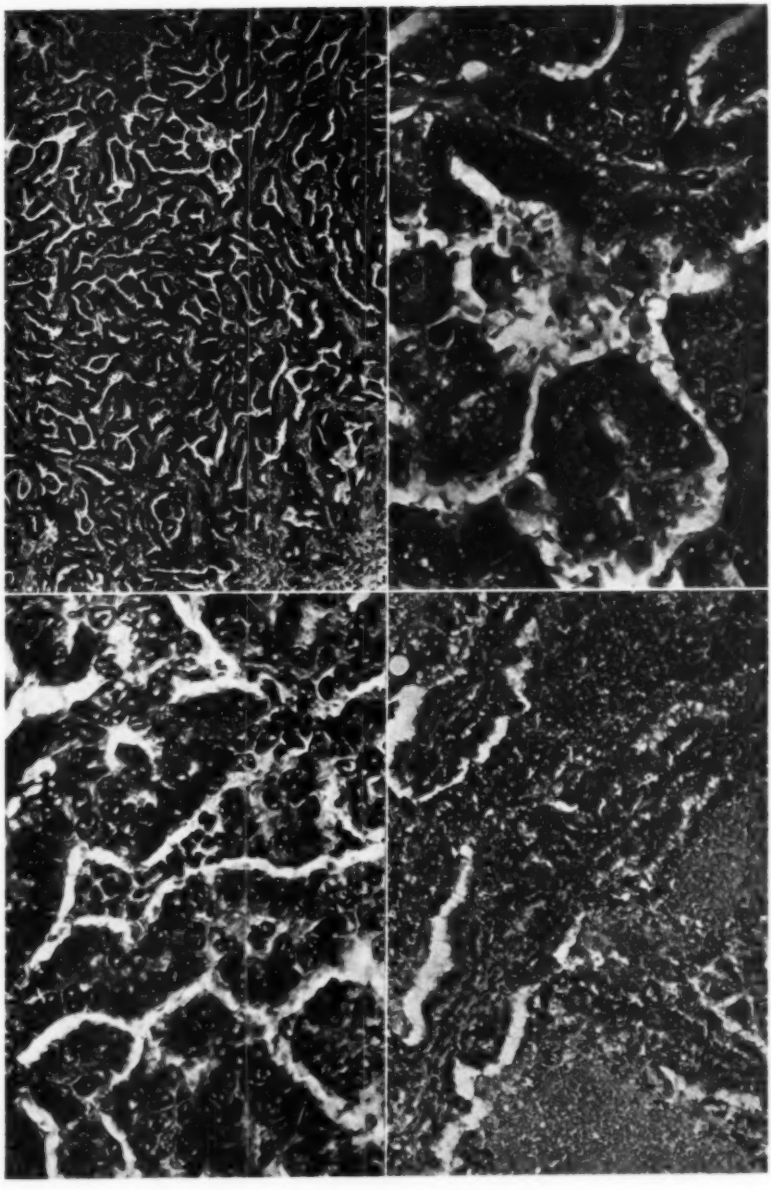

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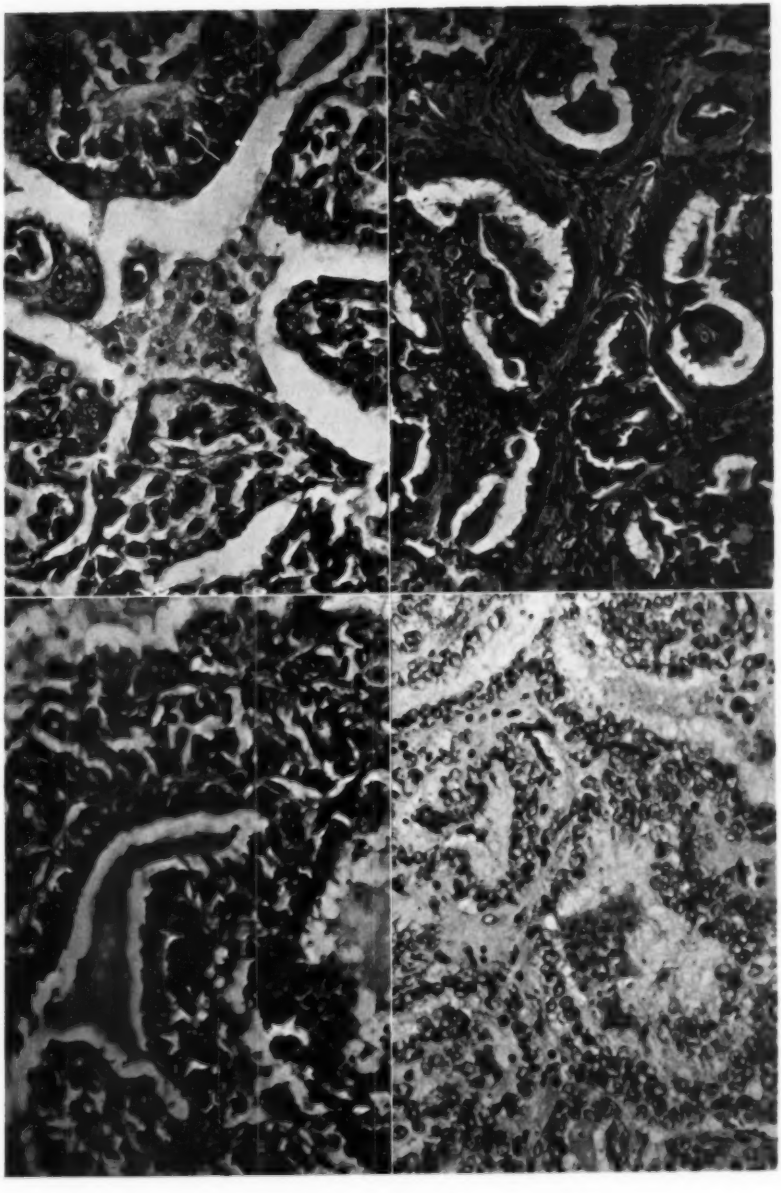

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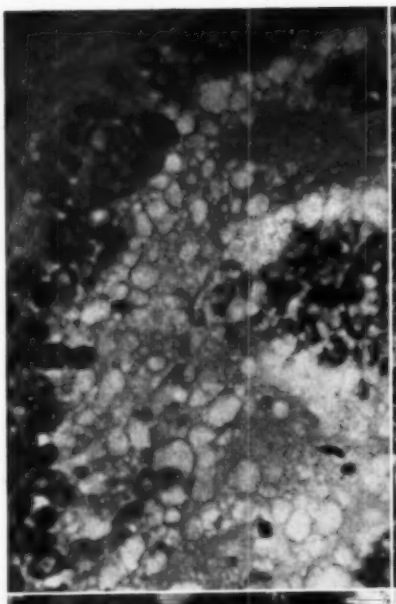

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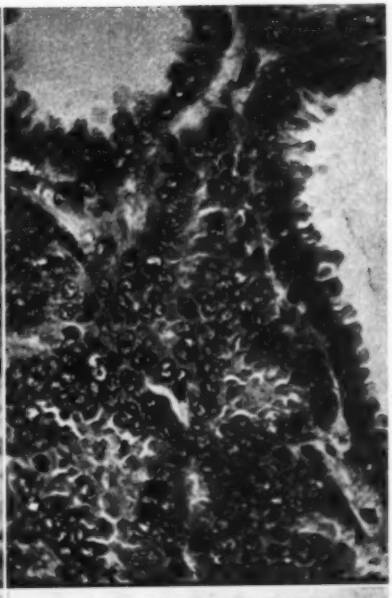

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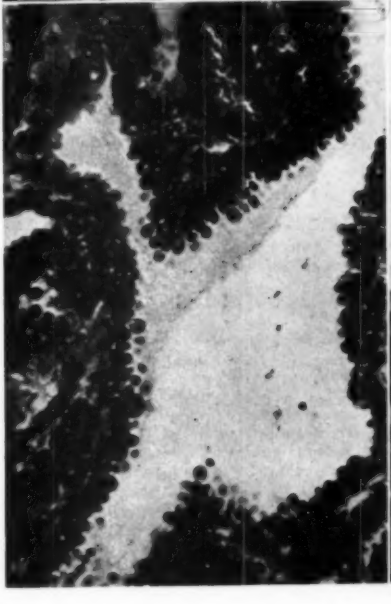

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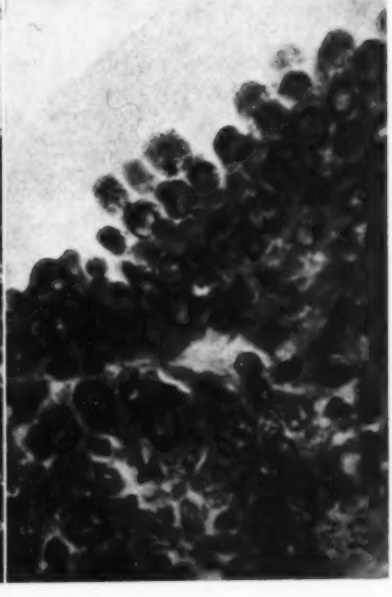

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cellular changes (digestion, necrosis, degeneration) can be followed. Cell digestion is prominent in Fig. 20 and 21. Note also the marked cellular multiplication going on (Fig. 20) with resulting crowding of the cells in the newly formed epithelium. Necrosis preceding lysis can be seen in Fig. 21, 22, 23 and 26. Necrosis covering large areas of the field and emergence of a newly organized columnar epithelium surrounding the area of necrosis sometimes may be very striking (Fig. 22). In such areas, one may notice a vacuolar change in the cells lying at the line of separation between the organizing epithelium and the mass of necrosed tissue undergoing liquefaction.

Liquefaction of cells preceded by keratinization can rarely be seen in the formation of the papillary cystic pattern (Fig. 24). The surviving lining cells usually show a tendency to flat or cuboidal cell transformation when in contact with fluid derived from the lysis of keratinized tissue. It appears that the composition of the fluid in the lumen exerts some influence on the orientation of the lining epithelium.

Watery vacuolation in the cytoplasm, as well as in the nucleus preceding cellular lysis, can frequently be seen forming a papilliferous pattern often resembling hyperplastic thyroid tissue, as shown in Fig. 25–27. Vacuolated fluid, with or without remnants of cell structures lying at the periphery of the lumen is frequently seen (Fig. 25) where new cells are replacing the lysing cells. In an area of rapid and extensive vacuolar changes, the whole thickness of the lumen can be seen primarily filled with vacuoles (Fig. 26 and 27). Vacuolation in the remaining peripheral cells, as seen in Fig. 27, gives evidence of a progressive lytic process. With cessation of the process, the rounding of the margin of the surviving epithelium follows, as shown in Fig. 25.

Sometimes remnants of cells in the process of lysis can be seen as protruding tongues of cytoplasm (Fig. 28) with or without any degenerative changes, simulating what is known as apocrine secretion. In some areas one may notice that the

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EXPLANATION OF PLATES—*cont.*

FIG. 23.—This photomicrograph demonstrates a remnant of a sheet of lysing tumor cells mostly surrounded by clear fluid. The peripheral surviving cells, where they are adherent to this sheet, show a tendency to take a spindle shape and lie parallel to the border of the papillae. × 450.

FIG. 24.—Lysis of cells beginning with keratinization in the formation of a cystic or cystic papillary pattern. × 280.

FIG. 25.—Another papillary pattern is shown here where the microscopic picture stimulates that of hyperplastic thyroid tissue. The clefts between the papillae are filled with acidophilic fluid resembling colloid with peripheral vacuolation. The lining cells of the cleft on the right show active vacuolar changes. Those on the left probably represent a phase of cessation of vacuolation, and the layer of vacuolated or clear area adjacent to these cells appears to be a result of amalgamation of vacuoles arising from pre-existing lining cells before their transformation into coagulable fluid, as in the center. × 190.

FIG. 26 and 27.—These two photomicrographs are taken from the same tumor. There is liquefaction following marked vacuolar changes of the cells involving cytoplasm as well as nucleus, better demonstrated at higher magnification in Fig. 27. In Fig. 26 necrosis of a sheet of cells can also be seen surrounded by vacuolated fluid. Fig. 26. × 290. Fig. 27. × 500.

FIG. 28, 29 and 30.—These photomicrographs are taken from the same tumor. Fig. 30 is a magnified view of the upper part of the preceding photomicrograph. In Fig. 28, the remnants of lysing cells lining the cavities still attached to the surviving cell layers form protruding tongues (apocrine secretion). In Fig. 29 and 30, the lysing cells are dropping off, as spheres in some of which fading nuclei can be recognized in earlier stages. The liquefied cells are being replaced by the newly formed cells originating in the deeper layers where a large number of mitoses are also seen. Fig. 28. × 405. Fig. 29. × 325. Fig. 30. × 900.

individual cells lining papillae or cystic cavities simply drop off in the form of spheres while lysing (Fig. 29, 30). Their place is taken up by new cells arising from the deeper layer. This mechanism has been known as holocrine secretion.

DISCUSSION

It is evident from this study that cell lysis is an active process associated with growth ; by that, we mean that cell lysis is not due only to aging of the cells Any one mass of cells comprises cells of different ages and different generations, but they are lysing in groups following various patterns. No vascular phenomenon such as obstruction or diseased vessels can be seen to explain this group lysis. Cell multiplication occurring side by side with lysis, either in diffusely scattered or in organized groups, also rules out the possibility of vascular damage as the cause of cell lysis. This lytic process can be noticed under the microscope in the tiniest tumor nodules. Active lytic processes were noticed not only in tumors but also in non-neoplastic tissues, epithelial or otherwise. This is more prominent in neoplastic tissue, as shown here and also in different disease conditions (Ghosh, 1957). We agree with Ewing (1940) that tumor cells in general suffer the same degenerative changes as occur in other tissues and also with Fischer (1935), who noticed the early disappearance of cancer cells by hydrolysis.

In this paper we have shown that an important consequence of cell lysis, as regards the utilization of space, is to make room for the multiplying tumor cells. We have tried to demonstrate here how cell lysis and cellular growth, following various courses and patterns, may contribute to the mechanism of formation of different histologic designs. In the past these designs were simply demonstrated without much attempt at explaining the mechanism of their formation and in addition, the proliferation of cells was always demonstrated but no tentative solution offered to explain the accommodation of this ever increasing number of cells. A histological section static as it is, is at the same time a cross section through a stream of developmental processes proceeding in time, as Weiss (1950) stated. Viewing every morphological criterion as a phase in a continuous chain of transformation should make it possible to reconstruct a sequence of events toward formation of different structural patterns present in the sections. It is hoped in the future, with the improvement of Transparent Chamber Technique, or with the perfection of tissue culture methods when various structural patterns in the organisms would be reproducible *in vitro*, that direct observations on differentiation may prove possible as well as the recording of the chain of transformation in the life history of a group of cells.

In the course of this study, cellular lysis and cellular growth have been seen to result not only in the formation and interchange of various morphologic patterns, but also in the maintenance of an apparently similar pattern in a state of dynamic equilibrium. Although the relative rates of cellular lysis and multiplication determine the compactness of cells in any one area, the actual rates of these processes represent the speed of the divergent biochemical reactions and their concurrent energy turnover, as well as the time period available to the cells for differentiation.

It appears that the lytic fluid has a possible role in the formation of new cells which replace it and in the organization of the peripheral cells forming its boundary. Carrel (1925) suggested that cancers grow because of excessive cell death and the

release of substances which not merely stimulate growth but also contain the food material necessary for the synthesis of protoplasm. Earle (1937) has reported the stimulating effect of tumor extracts on the multiplication of the same tumor cells. It has been stated (Ris, 1955) that the substances released by injured cells and tissue extracts contain a whole spectrum of factors, substrates, coenzymes, building blocks and possibly self-reproducing enzyme systems.

Several other histological tumor patterns which have been studied will be described in future communications in relation to the observations made on the formation of the connective tissue stroma and vascular system in the tumor.

Summary

In studying the developmental processes of mammary carcinoma of the mouse, active cellular lysis was found to be an important phenomenon. Among other things, it plays a prominent role in the accommodation of the multiplying cells and in the formation of different epithelial patterns. Preceding cellular lysis, there may not be any demonstrable cellular change or the cells may show various kinds of degeneration or necrosis. The role of cellular lysis in the enhancement of cellular multiplication is discussed on the basis of the present observations. In this study, dynamic processes such as cell lysis, movement of cells, shift of cell layers, in addition to true proliferation and change of cellular size and shape, are shown to take part in the differentiation and maintenance of various histologic patterns.

I am greatly indebted to Mrs. Dixie McGregor, Miss Lois Becker and Mrs. Rose Valle, who have assisted in various phases of this work, which was supported by the Copher Research Fund.

REFERENCES

Arey, L. B.—(1940) 'Developmental Anatomy'. Philadelphia and London (W. B. Saunders Co.)

Bailey, F. R. and Miller, A. M.—(1921) 'Textbook of Embryology', New York (William Wood & Co.)

Carrel, A.—(1925) *An. Surg.*, **82**, 1.

Earle, W. R.—(1937) *Arch. exp. Zellforsch.*, **20**, 140.

Ewing, J.—(1940) 'Neoplastic Diseases'. Philadelphia and London (W. B. Saunders Co.)

Fischer, A.—(1935) *Cancer, Brux*, **12**, 160.

Foulds, L.—(1956) *J. nat. Cancer Inst.*, **17**, 783.

Ghosh, H.—(1957) *Amer. J. Path.*, **33**, 598.—(1959) *Brit. J. Cancer*, **13**, 115.

Gierke, E.—(1908) *Sci. Rep. Imp. Cancer Res. Fd.*, **3**, 115.

Maximow, A. A. and Bloom, W.—(1957) 'A Textbook of Histology'. Philadelphia and London (W. B. Saunders Co.)

McMurrich, J. P.—(1920) 'The Developmebt of the Human Body'. Philadelphia (P. Blakiston's Son & Co.)

Murray, J. A.—(1908) *Sci. Rep., Imp. Cancer Res. Fd*, **3**, 69.

Ris, H.—(1955) 'Analysis of Development'. Philadelphia and London (W. B. Saunders Co.)

Weiss, P.—(1950) *Quart. Rev. Biol.*, **25**, 177.

EXPERIENCES WITH HYPOPHYSECTOMY IN MICE: HISTOLOGY OF PITUITARY REMNANTS

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HYPOPHYSECTOMISED mice have been used as test objects in many hormone research laboratories since 1933. In that year Collip, Selye and Thomson reported the results of experiments on animals which had been hypophysectomised by a technique similar to that previously used in rats by Smith (1926 and 1930). This involved a para-pharyngeal approach to the base of the skull. A dental burr was used to drill the bone on the inferior surface of the pituitary fossa and the gland was then removed by suction. The disadvantages of this operation were

(*a*) high post-operative mortality: and

(*b*) difficulty in obtaining complete removal of the gland.

Accordingly, Thomas, in 1938, devised another technique for which he claimed a lower mortality rate and which gave an improved exposure of the gland prior to removal. Briefly, Thomas' operation employs the same parapharyngeal approach as that of Smith but instead of using a dental burr to drill a hole through the bone, a quadrilateral panel is removed from the basi-occiput. This allows the operator a good view of the pituitary with a greatly enhanced opportunity for complete removal.

Hypophysectomised mice have been used in these laboratories for investigations into the mammogenic growth responses induced by the administration of various hormone combinations in addition to human urine, human blood serum and human pituitary extract (Hadfield and Young, 1956*a* and *b*, 1958*a* and *b*).

The results of such experiments may be invalidated if functional pituitary tissue has not been completely removed and some test must be applied to each of the animals before experimental conclusions can be reached. Of the tests available, the most reliable is the examination of serial histological sections of the entire region of the pituitary fossa. This paper describes the histological findings in a series of hypophysectomised mice examined in this way.

MATERIAL AND METHODS

Thomas' technique was used for hypophysectomising mice between the 22nd and 27th days of life. Body weight at operation was usually between 10 and 12 grams but animals weighing 9 to 14 grams were not excluded. They were maintained on diet 41b with added rolled oats and water *ad libitum* but without cortisone or antibiotics. Weights were recorded daily from the day of operation until death.

At the conclusion of the experiments the mice were killed by coal gas and skinned. The pelts were pinned out on wax and fixed in Bouin's fluid for determination of the mammogenic growth response (details of this method are given in

our original report, Hadfield and Young, 1956*a*). The carcasses were fixed in formol saline and the heads and abdomens were opened to allow penetration of the fixative. When this primary fixation was complete, the brains were removed without touching the skulls in the region of the pituitary fossae, the skulls were then secondarily fixed in formol sublimate for 24 hours. Decalcification in Perenyi's fluid was complete in three days. Each skull was trimmed coronally in front of, and behind, the pituitary fossa, as well as laterally and inferiorly. The resulting block of tissue measured 1·5 to 2 mm. in thickness by about 10 mm. in width and 7 mm. in depth. Paraffin sections were cut in a coronal plane, 5μ thick. Sixteen of the sections were mounted on each slide and every fourth section was examined—i.e., the pituitary fossa was examined at intervals of 20 μ. Four hundred and thirty two mice have been examined in this way.

RESULTS

The pars anterior and the pars intermedia bear a superficial resemblance to one another and the distinction between them can be a matter of some difficulty. It is important, therefore, to define the histological criteria used in their identification.

In coronal sections the pars anterior occupies much more space laterally than medially, while the pars intermedia and pars posterior lie centrally and above it. The latter are separated from the pars anterior by a cleft. It follows, therefore, the fragments found laterally are more likely to be pars anterior than pars intermedia, while fragments lying close to the mid-line may be either. The cleft separating pars anterior from pars intermedia is of importance for it means that an epithelial remnant attached directly to pars posterior is unlikely to be pars anterior (Fig. 1).

Whereas the pars anterior is a vascular structure and contains numerous thin-walled sinusoids, the pars intermedia is almost completely avascular. The sinusoids of the pars anterior are thin-walled and possess, here and there, the flattened nuclei of vascular endothelium. This gives the nuclear pattern of the pars anterior a more irregular appearance than that of the pars intermedia. The arrangement of cells, too, is different. The sinusoids of the pars anterior surround groups of cells which are quite irregular in size and shape, while cells and nuclei of the pars intermedia lie in approximately parallel columns (Fig. 2).

The eosinophils of the pars anterior, a prominent feature in sections stained by haematoxylin and eosin, are not found in the pars intermedia. This is the most important histological difference between pars anterior and pars intermedia and every fragment containing eosinophil cells was immediately designated pars anterior (Fig. 3).

The differences between pars anterior and pars intermedia are summarised in Table I.

Appearance of remnants of pars anterior

Most of the remnants of pars anterior consist only of a few cells. They are situated usually to one side, tend to be separated from pars intermedia and pars nervosa by a cleft, and often lack their typical histological structure. In most

TABLE I.—*Differences Between Pars anterior and Pars intermedia*

	Pars anterior	Pars intermedia
1	In coronal sections, more tissue seen laterally than medially	In coronal sections, most tissue is present medially.
2	Separated from pars posterior by cleft	Runs directly into pars posterior.
3	Vascular. Numerous thin-walled sinusoids	Avascular.
4	Irregular nuclear pattern due to presence of vascular endothelium	Nuclei tend to be regular in size and shape.
5	Irregular arrangement of epithelial cells	Cells in parallel columns.
6	Presence of eosinophils	No eosinophils.

cases they are recognisable by the presence of eosinophils (Fig. 4, 5, 6 and 7). Larger fragments possessing the typical histological picture of pars anterior are less common and are easily identified (Fig. 8 and 9).

Occasionally, groups of cells have been seen which appear to consist mainly of degenerating eosinophils. These are obviously fragments of pars anterior which have failed to survive after their accidental transplantation (Fig. 10, 11 and 12). Their presence may explain those cases of mice whose initial gain in weight after operation suggests incomplete removal but which do not continue to grow after the first week or so.

On several occasions a fragment of pars intermedia has been seen in which the unusual presence of blood vessels close to the end of the fragment has led to an atypical appearance and to the possibility of confusion (Fig. 13). The capillaries extend only a short distance into the fragment and sections taken some way through the block show a perfectly normal appearance of pars intermedia.

Whereas the principal tissue liable to be confused with pars anterior is pars intermedia, it is not in fact the only one. Within, or near, the fibrous scar replacing the removed occipital bone, one sometimes finds another structure which might be mistaken for pars anterior, especially if the latter were degenerating. This is the brown adipose tissue. Its fat content and appearance vary with the state of nutrition and physical condition of the animal. When much fat is present, there is no possibility of confusion but when fat content is low, a resemblance exists.

The adipose tissue does not, however, occur within the skull. It lacks the typical eosinophil cells of the pars anterior and, owing to differences in nuclear size and shape, it has a considerably more variegated appearance even than pars anterior. It can be found in intact animals and in animals which have undergone a " sham " operation in which all steps in the operation for hypophysectomy are carried out short of removal of the hypophysis. It can also be found without opening the skull, and " signet ring " cells, which are common in this tissue, have never been seen in genuine pituitary remnants (Fig. 14 and 15).

Position of remnants

Most pituitary remnants are found inside the skull, near the mid-line, and immediately above the synchondrosis separating basi-sphenoid from basi-occiput.

(*a*) Fragments are not usually found outside the skull but their occurrence in this position may be due to :

i. Small groups of cells becoming embedded in the fibrous tissue which replaced the quadrilateral flap of basi-occiput removed at operation.

These apparently arise from small isolated fragments lying loose and becoming incorporated in the blood clot which forms immediately the bone flap is removed (Fig. 16 and 17).

ii. Continuity with residual larger fragments in the pituitary fossa. Serial sectioning has shown that the two fragments are, in fact, continuous, and a connecting bridge of tissue can be traced running round the cut surface of the synchondrosis (Fig. 18 and 19).

(*b*) Fragments of pars nervosa and intermedia are most commonly found on or near the midline, for in this area they have their maximum thickness. On the other hand, pars anterior has its greatest thickness some distance lateral to the midline (Fig. 1). For this reason fragments of pars anterior are often found laterally although they may occur centrally as well.

(*c*) In the majority of our cases pituitary remnants, when present, are found just above the synchondrosis. It appears that the action of cutting the cartilage causes a roughness or irregularity which is liable to lead to retention of small fragments of pituitary.

The operation devised by Thomas (1938) does not give *complete* exposure of the pituitary gland in our strain of mice. In spite of this, it is quite exceptional for large pituitary fragments from the anterior portion of the gland to be found under the intact basi-sphenoid.

Size and frequency of occurrence of remnants

No attempt has been made to measure the size of the anterior pituitary remnants found other than by a subjective grading of + : ++ : +++ Criteria used for this are as follows :

\+ = Fragments containing only a few cells. Histological structure is not necessarily typical but the tissue is recognisable by the presence of eosinophils.

++ = Somewhat larger fragments than the above in which histological structure of the normal pars anterior is present.

+++ = Fragments which appear to comprise a substantial part of the whole gland—possibly accounting for 10 per cent. or even more.

TABLE II.—*Frequency of Occurrence of Different Types of Pituitary Fragments Found on Examination of Four Hundred and Thirty Two Mice*

Pituitary fragments found in :	Number of mice	Percentage of total
Pars anterior +	67	15·51
++	6	1·39
+++	0	0
Pars intermedia	182	42·13
Pars posterior	400	92·59

DISCUSSION

1. The frequency with which fragments of pars anterior are found depends to a great extent on the care with which the operation is performed. Most of the first groups of mice examined histologically were found to contain fragments of

pars anterior. At the risk of killing the mouse by tracheal compression and asphyxia, longer suction was applied during the operation, especially to the lateral corners of the pituitary fossa. Subsequent histology showed that complete removal was then the rule rather than the exception.

2. Although large fragments of pars anterior are uncommon, it is an easy matter to leave small groups of cells behind. Since these are often situated within the skull in an irregularity on the synchondrosis, the Thomas operation has been modified slightly and the synchondrosis is removed along with the panel of basi-occiput. This gives even better access to the pituitary gland which is now, in our mice, almost completely exposed to view. In mice of less than 10 grams weight this modification appears to result in higher operative mortality but heavier mice tolerate the operation well.

In an interesting paper published in 1955, Lostroh and Jordan describe their hypophysectomy technique in which the basi-occiput is penetrated by a dental burr. This is a return to the procedure used by Smith (1926, 1930) and by Selye, Collip and Thomson (1933). In view of the ease with which fragments can be left behind, even with the relatively full exposure of the Thomas operation, it is difficult to see how complete removal can be effected through a small drill hole with sharp edges.

EXPLANATION OF PLATES

FIG. 1.—Coronal section through pituitary gland of normal 28-days-old male mouse. P.T.A.H. × 50.

FIG. 2.—Pituitary of normal 28-days-old mouse to show pars intermedia and pars anterior (below) separated by cleft. H. and E. × 450.

FIG. 3.—Pituitary of normal 28-days-old mouse stained to show eosinophils in pars anterior. P.T.A.H. × 450.

FIG. 4.—A fragment of pituitary consisting of pars posterior, pars intermedia and a few cells of pars anterior with a small residual " cleft ". H. and E. × 50.

FIG. 5.—High power view of Fig. 4 to show the presence of eosinophils. H. and E. × 450.

FIG. 6.—A fragment of pituitary consisting of pars posterior, with groups of cells from pars intermedia and pars anterior with residual cleft. H. and E. × 50.

FIG. 7.—High power view of Fig. 6 to show the presence of eosinophils. H. and E. × 450.

FIG. 8.—A fragment of pars anterior situated laterally in the pituitary fossa. H. and E. × 50.

FIG. 9.—High power view of Fig. 8 to show characteristic structure of pars anterior. H. and E. × 450.

FIG. 10.—Fragment of pituitary containing pars posterior centrally and pars anterior lateral to it. H. and E. × 50.

FIG. 11.—High power view of Fig. 10 to show atypical degenerating eosinophils. H. and E. × 450.

FIG. 12.—Fragment of pars anterior isolated in scar tissue, degenerating. H. and E. × 450.

FIG. 13.—Unusual appearance of pars intermedia due to parallel capillaries. H. and E. × 450.

FIG. 14.—" Brown adipose tissue " showing variegated nuclei and presence of " signet-ring " cells. H. and E. × 450.

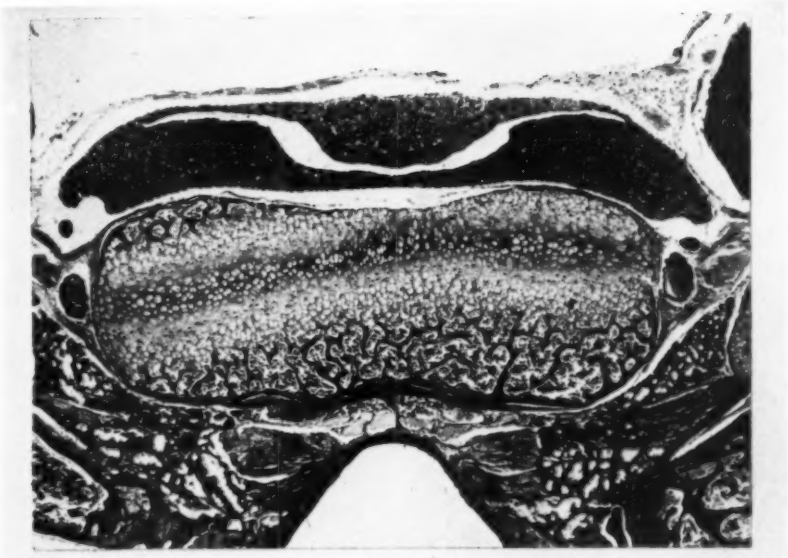
FIG. 15.—" Brown adipose tissue " simulating appearance of pars anterior. H. and E. × 450.

FIG. 16.—Isolated fragment of pituitary lying centrally within fibrous scar. H. and E. × 50.

FIG. 17.—High power view of Fig. 16 showing pars intermedia (above), cleft, and pars anterior (below). H. and E. × 450.

FIG. 18.—Two fragments of pituitary tissue, apparently isolated from one another. H. and E. × 50.

FIG. 19.—Two fragments of pituitary tissue, apparently isolated from one another, but showing connecting " bridge " of tissue. H. and E. × 50.



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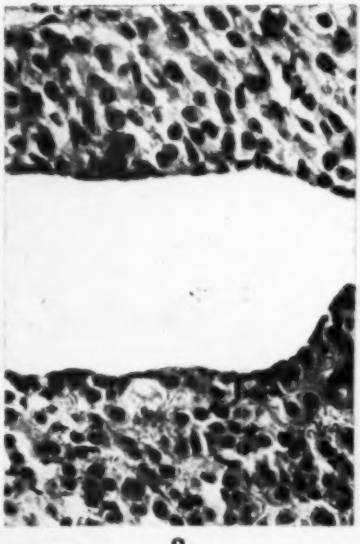

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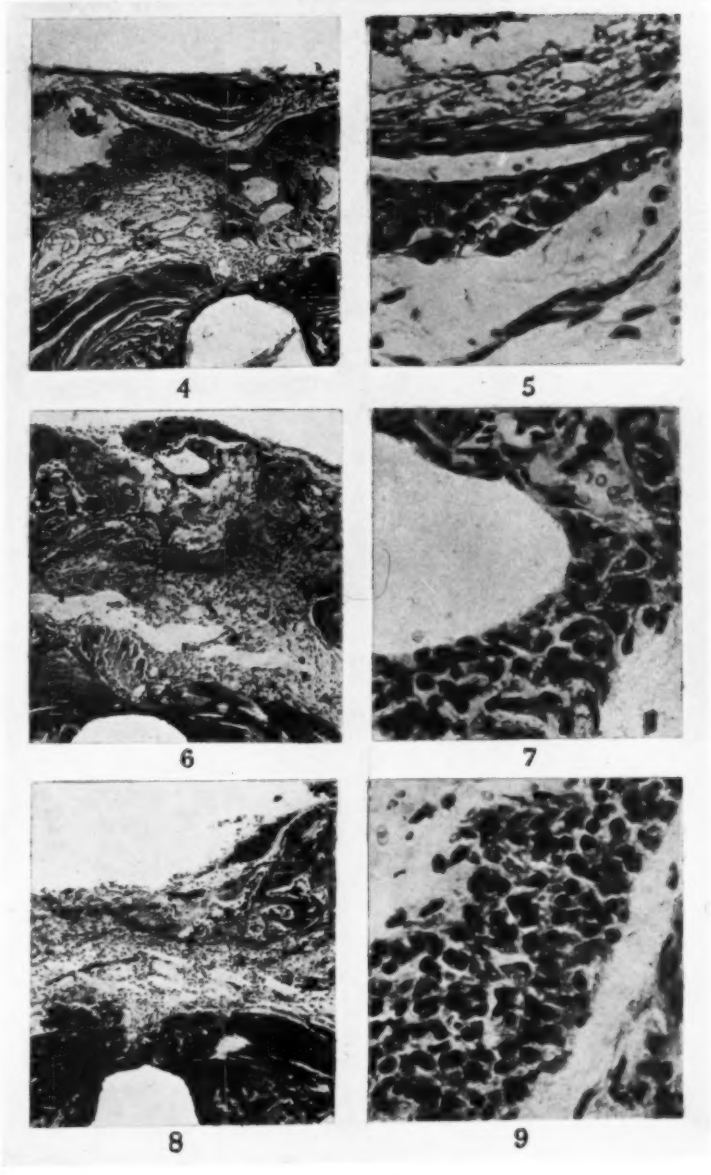

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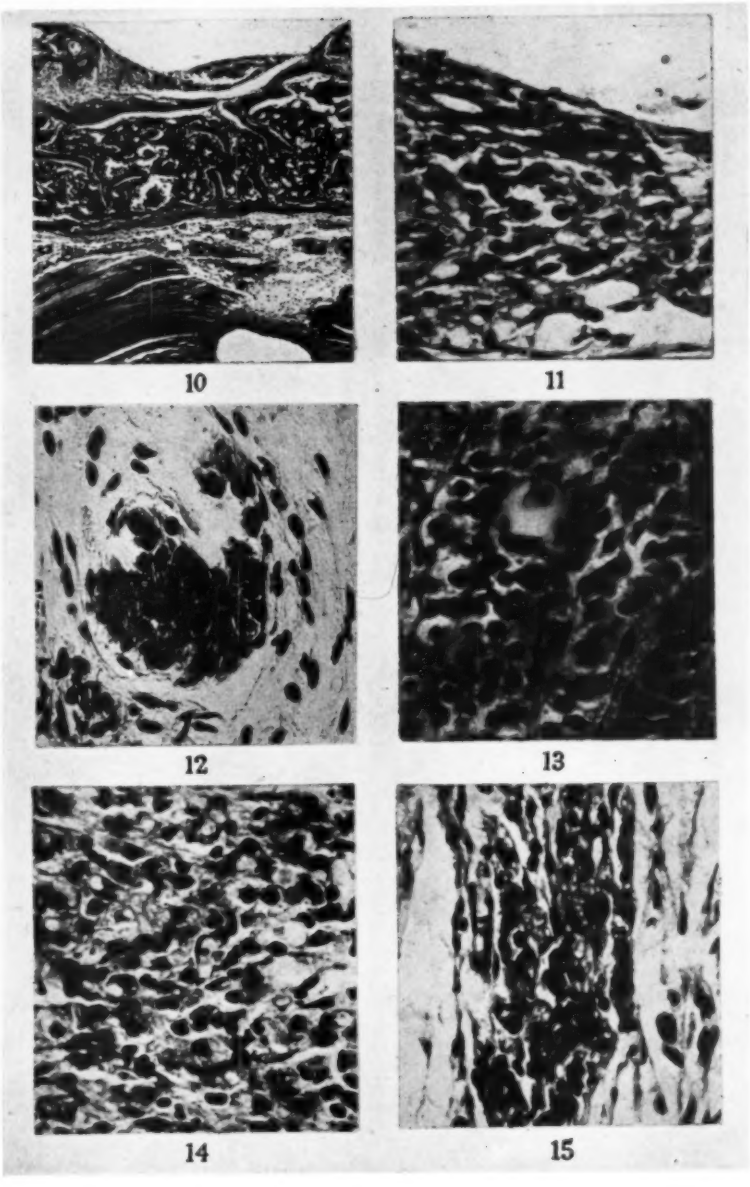

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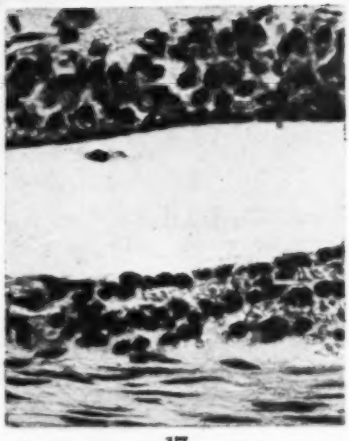

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The infrequency of large fragments of pars anterior occurring in this series is in contrast to the histological findings of Bahner and von Graff (1957) who reported fragments of size II or larger in 14 out of their 25 animals. With a larger series of four hundred and six mice and judging completeness of hypophysectomy by the loss of weight, (and hence the degree of atrophy) of the seminal vesicles, they concluded, however, that about 20 per cent of their animals possessed active remnants. This is a similar figure (if somewhat larger) to the 16·8 per cent found in the present series by purely histological means.

Fragments of pars nervosa or " pseudo-stalk " were common in both sets of cases.

SUMMARY AND CONCLUSIONS

1. Serial histological sections have been cut through the pituitary fossae of four hundred and thirty two mice hypophysectomised by the technique of Thomas (1938) and the results have been studied.

2. Complete hypophysectomy—i.e. to include pars anterior, intermedia and posterior—is very difficult to achieve.

3. Large fragments of pars anterior are not common but small ones, amounting to only a few cells, are present in about 16 per cent of the animals studied.

4. Fragments are usually left in the region of the synchondrosis.

5. The operation has been modified to allow removal of the synchondrosis and a more complete view of the pituitary gland.

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REFERENCES

Bahner, F. and von Graff, Hedy.—(1957) *Acta endocr., Copenhagen*, **24**, 333.
Collip, J. B., Selye, H. and Thomson, D. L.—(1933) *Virchows Arch.*, **290**, 23.
Hadfield, G. and Young, S.—(1956*a*) *Brit. J. Cancer*, **10**, 145.—(1956*b*) *Ibid.*, **10**, 324.—(1958*a*) *Lancet*, i, 568.—(1958*b*) *Brit. J. Surg.*, **46**, 265.
Lostroh, A. J. and Jordan, C. W.—(1955) *Proc. Soc. exp. Biol., N.Y.*, **90**, 267.
Selye, H., Collip, J. B. and Thomson, D. L.—(1933) *Ibid.*, **31**, 82.
Smith, P. E.—(1926) *Anat. Rec.*, **32**, 221.—(1930) *Amer. J. Anat.*, **45**, 205.
Thomas, F.—(1938) *Endocrinology*, **23**, 99.

STUDIES ON THE INDUCTION OF BONE AND SOFT TISSUE TUMOURS IN RATS BY GAMMA IRRADIATION AND THE EFECT OF GROWTH HORMONE AND THYROXINE

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KNOWLEDGE of the dose of X- or gamma rays sufficient to induce malignant tumours in bones of humans and experimental animals is of practical and theoretical importance. Data on this subject are surprisingly limited and almost all of our information is based on not more than about 70 published cases of bone tumours in man (Sabanas, Dahlin, Childs and Ivins, 1956) and on very scanty and much less enlightening data from experimental animals. All of the bone tumours known to have been produced by external irradiation in human beings have occurred as a result of radiation therapy. The exact tissue dosages in most instances are not known, but it is apparent that all cases occurred after relatively large doses. The published figures range from 1400 to 10,000 roentgens (Cahan, Woodard, Higinbotham, Stewart and Coley, 1948 ; Jones, 1953 ; Sabanas, *et al.*, 1956).

The data on bone tumours produced by external irradiation of experimental animals are even more limited than the human data. Four malignant bone tumours have been reported after local irradiation of rats and rabbits with X-rays (Marie, Clunet and Roulot-LaPointe, 1910 ; Lacassagne and Vinzent, 1929 ; Ludin, 1930 ; and Lacassagne, 1933). The dosages again were high, but it is not possible to make reliable estimates of the tissue dosages in those experiments. In addition, five osteogenic sarcomata have been observed by Koletsky and Gustafson (1955) in a series of 123 Wistar rats exposed to 660 r of whole-body X-irradiation (220 KVp.). No other data have been found in the literature with regard to the production of bone tumours by either local or whole-body exposure to X- or gamma rays.

An estimate of the true tissue dose in the bones of either animals or human patients exposed to conventional X-rays (220 KVp.) is made difficult by the fact that the energy absorbed in bone is about 2 to 3 times greater than that in the soft tissues for the same dose of roentgens (Spiers, 1949). It is likely, therefore, that the radiation doses received by the susceptible cells in the bones of many of the cases under discussion were considerably greater, in general, than those cited in the respective papers. The prevailing uncertainties regarding the dose required to produce malignant bone tumours in experimental animals with external irradiation and the absence of any morphological studies concerning the patho-

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genesis of neoplasms under such conditions made it desirable to study this problem in some detail.

Accordingly we endeavoured to produce bone tumours in rats by local irradiation with a relatively large single dose of gamma rays. Gamma rays were chosen in order to avoid the aforementioned difficulties in dose estimation that are encountered with conventional X-rays. A radio-iridium source was available for the purpose. The absorption of gamma rays from iridium ^{192} is only about 10 per cent higher in bone than in soft tissue (as calculated from the data of Spiers, 1949). A dose of 3000 roentgen was chosen because, on the basis of clinical and experimental data (Woodard and Coley, 1947; Barr, Lingley and Gall, 1943; Sissons, 1956), it was thought that this dose would be large enough to produce severe and permanent tissue damage without at the same time causing excessive bone necrosis, fractures and other complications leading to premature death of the animals. It also seemed desirable to use a dose level somewhat comparable to the human exposures and it can be seen that 3000 r is just about twice as large as the lowest reported figure in the data on post-irradiation sarcoma in man (Cahan *et al.*, 1948). Although it would have been desirable to study the effects of at least two different dose levels in sufficient numbers of animals, this proved impractical. However, it was thought to be feasible to investigate simultaneously the subsidiary question whether hormone treatment of animals following irradiation would have a measurable effect on the induction time and yield of tumours. Bone growth and metabolism are known to be influenced by a number of hormones and since abnormalities in hormonal balance are also known to be important factors in the genesis of some tumours in other organ systems it was decided to study the influence of two hormones on the induction of tumours. The two hormones chosen for experimentation have a different and fairly specific action on skeletal tissue: (1) growth hormone stimulates the growth of cartilage (Evans, Becks, Asling, Simpson and Li, 1948) and periosteal osteogenesis (Becks, Asling, Simpson, Li and Evans, 1949); (2) thyroxine acts on the turnover of bone, increasing the excretion of calcium and phosphorus, and producing morphological evidence of excessive bone resorption (Follis, 1953).

METHODS

Female Wistar rats, 60 days old and weighing between 100–130 g. were irradiated with 3000 r to both knee joints from a Radio-Iridium teletherapy unit (Freundlich and Haybittle, 1953) emitting primary gamma rays with a mean energy of 0·38 Mev. half-value layer 8·1 mm. Cu. (see Fig. 1). The dose rate varied on different occasions from 35 to 42·5 r per minute, requiring an exposure of 70 to 85 minutes. A perspex frame was made on which the anaesthetized rats could be fixed by bandage slings. Four rats were exposed simultaneously and a special lead and perspex applicator was constructed so that the 4 pairs of knee joints comprising the lower half of the femur and upper half of the tibia would be irradiated (Fig. 2). The animals were anaesthetized with either nembutal (0·1 ml./100 g. I.P.), or with sodium amytal (7 mg./100 g. I.P.). The radiation was completed in 8 sessions all performed within a period of 15 days.

One hundred and thirty six (136) irradiated rats were divided into 4 groups, A, B, C and D, and each group shared as evenly as possible the rats from the 8 radiation sessions. 34 rats from the same litters were allocated to the unirradiated

control groups, E, F, G and H. Eighty days after irradiation, hormone injections were begun.

Group A of 31 rats and control Group E of 5 rats were given daily injections (except Sunday) of growth hormone subcutaneously in the flank in increasing doses : 30 μg. per rat per day for the 1st week ; 60 μg. for the 2nd week ; 120 μg. for the 3rd and the 4th week ; 240 μg. for the 5th and 6th week ; 360 μg. for the 7th week ; 480 μg. for the 8th week ; 780 μg. for the 9th week and 1 mg. for the 10th week (a total of 20·58 mg. per rat in 10 weeks). The growth hormone used was Somacton for 6 weeks, and for the last 4 weeks was a preparation made by the Wilson Laboratories, Chicago, Illinois.

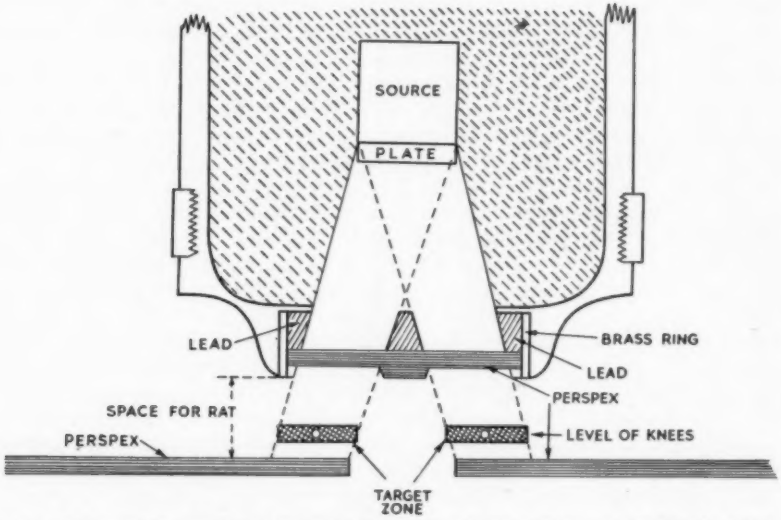


FIG. 1.—Section of the radium-iridium teletherapy unit (Freundlich and Haybittle, 1953) showing the gamma-ray source, the lead applicator, and the perspex frame to which the rats were fastened.

Group B of 30 irradiated rats and Group F of 5 control animals were given daily (except Sundays) subcutaneous injections into the flank of Na 1 Thyroxine (Glaxo Laboratories Ltd.), dissolved in physiological saline containing 0·01 M sodium carbonate, 20 μg. per rat per day for 3 weeks, 30 μg. for another 3 weeks, and 45 μg. for the last 4 weeks, making a total of 1·98 mg. per rat (10 mg./kg.), over the 10 weeks.

Group C of 28 irradiated rats and Group G of 5 control rats were given a 10 weeks course of growth hormone injections identical with Groups A and E, and followed by a 10 weeks course of thyroxine injections identical with Groups B and F. Group D of 37 irradiated rats were injected daily with saline, half the group were injected for 10 weeks and half for 20 weeks. A control group H of 19 unirradiated rats were injected with saline, half for 10 weeks and the remainder for 20 weeks.

All rats were weighed once a week and growth curves plotted for each cage and each group.

Diagnostic X-rays were made of the legs and lower abdomen of each rat at 185, 280 and 386 days. The rats were anaesthetized with ether and rapidly positioned on the X-ray plate with cellotape. Both knees of each rat were palpated at least once a week and the occurrence of tumours noted. As soon as it was certain

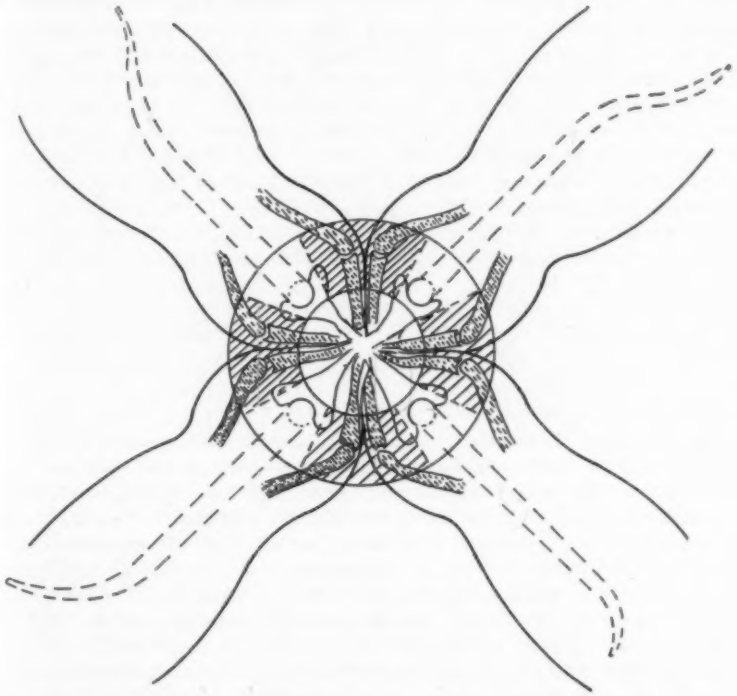


FIG. 2.—Plan of the 4 rats fastened to the perspex frame by slings of bandage. The shaded zones are the ports in the lead applicator. Both knee joints and 1 cm. of the adjacent femoral and tibial shafts of each rat were irradiated.

that a tumour was rapidly growing the animal was killed, a radiograph of the whole animal was made, and autopsy performed. Both hind-legs of each rat were fixed in formalin and after dissection they were decalcified with 3 per cent nitric acid, embedded in celloidin, sectioned and stained with haematoxylin and eosin. Other tissues, including the endocrine glands, were fixed in Suza solution and embedded in paraffin. A few of the controls were killed from time to time to have animals of comparable age to those of the irradiated groups, but most of the controls were killed when all irradiated animals had died.

RESULTS

1. *Clinical observations on irradiated animals*

All the irradiated rats showed complete depilation of the irradiated areas which persisted for some weeks. In some cases, this went on to ulceration in the skin creases. Discharging sinuses developed in a few rats which were later found to have extensive destruction of the bones of the knees with loss of the femoral epiphysis and a part of the femoral shaft. All these early lesions healed in time, but it was obvious that the dose of radiation could have not been increased much above 3,000 r without the danger of localised destructive lesions in the majority of the rats. There were some pathological fractures in animals after they had developed bone tumours. Most of the skin tumours described below developed in ulcers. These late ulcerations of the irradiated areas arose *de novo* and rapidly took the form of typical malignant ulcers. Most of these proved on histological examination to be epidermoid carcinomata, but some were sarcomata arising in soft tissues.

The injections of growth hormone were tolerated without ill effects. Animals injected with thyroxine were distinctly ill and their fur was ruffled, and although they did not lose weight the dose of thyroxine was not raised any further for fear of losing animals.

The mean survival times of the irradiated rats were as follows: Group A (growth hormone) 513 days; Group B (thyroxine) 504 days; Group C (growth hormone and thyroxine) 461 days; and Group D (saline) 494 days.

2. *The effect of growth hormone on the weight of the rats*

The mean weight of the rats at the time of irradiation was 118–120 g. At the commencement of the injection treatment 80 days later it had increased to 200–205 g. and the weight curves were beginning to plateau. In the 10 weeks of the course of injections the 33 irradiated rats injected with saline (Group D) showed a mean increase of weight of $19{\cdot}78 \pm 1{\cdot}7$ g. per rat. The 30 rats irradiated and treated with growth hormone (Group A) increased by $26{\cdot}7 \pm 1{\cdot}77$ g. The growth hormone had caused a small but significant increase in weight (Student's $t = 2{\cdot}79$, $n = 61$, $p < 0{\cdot}01$). Group C were also treated with growth hormone for 10 weeks and gained a mean of $25 \pm 1{\cdot}65$ g. per rat ($t = 2{\cdot}15$, $n = 59$, $p < 0{\cdot}05$). The 18 non-irradiated rats of Group H (injected with saline) increased by $22{\cdot}05 \pm 1{\cdot}88$ g. per rat over the same period, while the non-irradiated rats injected with growth hormone, Groups E and G, increased by $32{\cdot}2 \pm 3{\cdot}4$; significantly more than the controls, ($t = 2{\cdot}13$, $n = 26$, $p < 0{\cdot}05$). Thus the growth hormone injection caused a small but significant increase of body weight compared with the rats receiving saline injections in both the irradiated and non-irradiated rats. Group *B* (irradiated rats injected with Na 1 thyroxine) increased by $23{\cdot}1 \pm 1{\cdot}9$ g. per rat; this was not significantly different from the saline injected rats.

3. *Radiological changes in the bones occurring in the field of irradiation*

The X-ray pictures which were made 185 days after irradiation always showed some rarefaction of bone with thinning of the cortex. There was retardation of bone growth and the irradiated bones were always considerably shorter than the non-irradiated controls. The epiphyseal plates were widened in some animals, and occasionally detachment or complete destruction of an epiphysis occurred. At

280 days these changes were usually more pronounced. In 18 of the 116 irradiated survivors one or more transverse bony bars could be seen in the femoral shafts. Fusion of some epiphyses had occurred. At 386 days there was evidence of repair of these lesions and of recalcification of the bones in some animals. Twelve rats had already developed osteosarcomata by this time. Details of the X-ray and histological changes found in the irradiated bones will be the subject of another communication.

4. *The incidence and induction time of tumours*

The overall incidence of malignant tumours in all experimental animals and in the controls and the induction time of each are given in Fig. 3. It should be understood that the present classification of mesenchymal tumours into two groups, osteosarcomata and fibrosarcomata, is purely descriptive. A better classification may have been obtained by dividing all mesenchymal tumours according to their site of origin, i.e. bone, periosteum soft tissues. Unfortunately, the large extension of most of the tumours and their rapid growth, made such classification impossible or, at best, arbitrary. Under the circumstances, it was decided to adhere to a descriptive classification, without prejudice to the true site or origin of each tumour. However, it can be stated, with a reasonable degree of certainty, that all osteosarcomata originated in bone, and that all fibrosarcomata, with the notable exceptions mentioned below originated outside the bone, either from the periosteum or the soft tissues.

Osteosarcomata.—A total of 38 separate osteosarcomata were found in 34 rats out of a total of 116 irradiated animals. The incidence was higher in the animals treated with growth hormone, namely 12 out of 30, as compared with 7 out of 31 in the saline-injected animals. The incidence was four times higher in the femur than in the tibia. The shortest latent period was 251 days (Group B) and 252 days (Group A, see Fig. 3). In 12 rats osteosarcomata were already present before 386 days (3rd diagnostic X-ray). Fig. 4 shows the diagnostic X-ray at 386 days of an early osteosarcoma, and Fig. 5 shows the same tumour at post-mortem. The data on the incidence and mean induction period of osteosarcomata and fibrosarcomata are summarized in Table I. Note that the mean induction period of osteosarcomata in Group B (thyroxine-treated) is significantly shorter than in the other groups. ("d" test of Sukhatme (1938) significant at the 5 per cent level.) The mean induction period of fibrosarcomata is the same in all groups which adds significance

TABLE I.—*Incidence of osteosarcomata and fibrosarcomata after irradiation and hormone treatment*

	Osteosarcoma			Fibrosarcoma		
Treatment	Incidence	%	Induction period	Incidence	%	Induction period
Group A, γ rays + growth hormone	12/30	40	520±49	12/30	40	465±36
Group B, γ rays + thyroxine	8/29	27·5	388±29	9/29	31	468±33
Group C, γ rays + growth hormone and thyroxine	7/26	27	512±35	12/26	46	445±50
Group D, γ rays + saline	7/31	22·5	521±46	17/31	54·7	441±25
Totals	34/116	29·3	488±44	50/116	43	453±36

to the finding of a shortened latent period for osteosarcomata in Group B. No osteosarcomata or fibrosarcomata were found in the unirradiated controls.

The chance of a rat developing a bone sarcoma following irradiation increases steadily the longer the rat survives. Of the rats which survived more than 200 days after irradiation many died or had to be killed because of sarcomata of soft tissues or carcinomata of skin or mammary gland. Five rats in Group A and 6

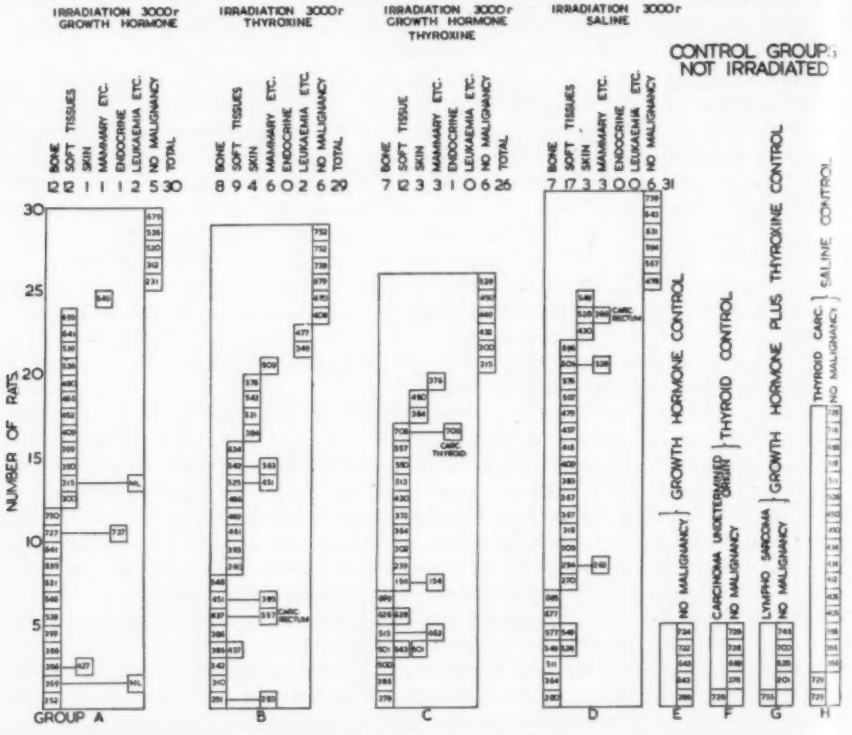


FIG. 3.—The incidence and induction time of malignant tumours of all types induced by irradiation and hormone treatment. Each square represents one rat, the number in the square is the time from irradiation to the detection of the tumour (in days). Note some rats had more than one type of tumour.

rats in each of Groups B, C, and D had no malignant tumours at death. Of these 23 rats, 9 had pneumonia or pasturella infection, 5 were killed because of very large fibro-adenomata, 2 had urinary infection, 2 pyometria, 3 pathological fractures, 1 intestinal obstruction and in 1 no obvious cause of death was found. The times of these non-specific deaths can be seen in Fig. 3. A life table analysis was therefore applied to the 4 groups of rats to compare :—

(1) The chances of developing a bone sarcoma ;
(2) The chances of developing a bone sarcoma or a sarcoma of soft tissue ;
(3) The chances of developing any malignant tumour at the site of irradiation.

A rat was at risk until it developed a tumour of the kind being studied or until it died from some other cause. Fig. 6 shows the percentage of rats at risk developing bone sarcomas. Note that 25 per cent of the rats at risk in Groups A, B and C have developed an osteosarcoma when 10 per cent of the rats at risk in Group D

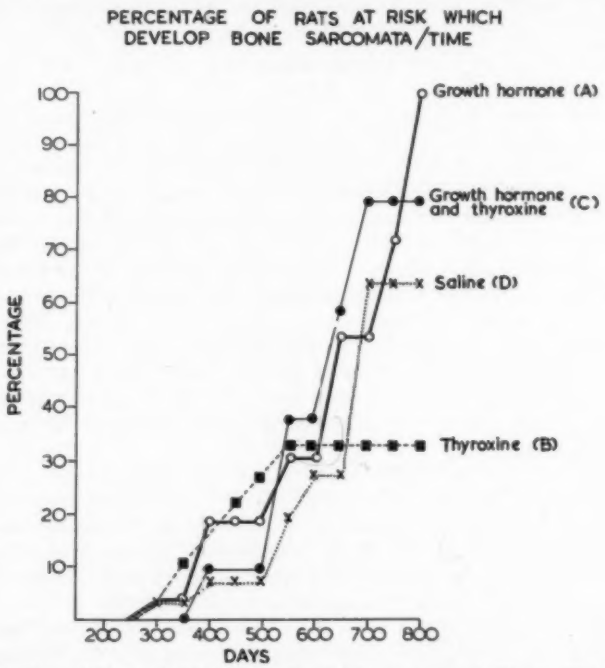


FIG. 6.—Percentage of "rats at risk" which develop bone sarcomata/time. The lower part of the graph indicates that the growth hormone and thyroxine treated rats develop bone sarcomata more quickly, the upper part of the graph is based on very few animals and is therefore of less significance.

have done so. The upper part of the graphs are based on very few animals and are less reliable. More details of these life-analysis data are given in Tables II and III.

Description of osteosarcomata.—The osteosarcomata ranged in size from microscopic tumours to large masses up to 40 mm. in maximum dimension. The 13 osteosarcomata found on microscopic examination were all limited to the bone, but they passed freely through the epiphyseal cartilages from the shaft to the

TABLE II

Percentage of rats at risk which developed	Treatment A	B	C	D
(i) Bone sarcomata in 550 days	33·4	30·5	38·1	19·4
(ii) Sarcomata of the bone or soft tissue in 400 days .	32·0	24·7	28·6	29·0
(iii) Malignant tumours after 400 days . . .	35·6	31·8	41·1	35·5

TABLE III

Time taken in days for	Treatment A	B	C	D
(i) 30 per cent of rats at risk to develop bone sarcoma . .	548	525	536	654
(ii) 50 per cent of rats at risk to develop sarcomata of bone or soft tissue	512	517	531	467
(iii) 50 per cent of rats at risk to develop malignant tumours .	467	494	451	488

epiphysis or *vice versa*. They were all of the so-called "sclerosing type", with disorderly arranged, irregular trabeculae, surrounded by a highly cellular tissue with atypical cells. In all the other instances the tumours spread beyond the bone, but only 6 of them were typical sclerosing osteosarcomata (Fig. 7 and 8), while 6 were very cellular tumours that could be recognized as osteosarcomata only because of the occasional presence of small spicules of bone. The other 14 tumours had a consistent pattern; the central portion showed obvious bone formation but the peripheral portion consisted of a richly cellular tumour tissue that could easily be mistaken for a sarcoma arising in soft tissue. There was often a sharp boundary when one pattern passed into the other (Fig. 9).

Metastases were found in the aortic lymph nodes and lungs of 5 animals: 2 in Group A, and one in each of the other groups. Fig. 10 shows a typical example of a metastasis in lung. In two cases, the metastases were more differentiated than the primary tumours.

Fibrosarcomata.—The term Fibrosarcoma, as mentioned above, embraces all the sarcomata that did not produce bone. Most of them were fairly well differentiated fibrosarcomata (Fig. 11) although 7 were anaplastic, undifferentiated and markedly pleomorphic (Fig. 12). In these instances, it was impossible to determine with certainty the site of origin of these tumours, but since they did not produce bone, they were arbitrarily classified as fibrosarcomata.

The 60 fibrosarcomata which were found in 50 animals ranged in size from a microscopic tumour (arising in or near the periosteum of a rat of Group C killed at 706 days) to large masses, up to 55 mm. in maximum dimension. In 9 instances, the tumours were in the subcutaneous tissue, in all other cases the tumours had invaded the muscles and tissues adjacent to the bones. The femur was replaced by tumour in 7 cases, the tibia in one, and in other instances there was invasion of the articular cartilage or replacement of the patella.

Other neoplastic conditions.—An interesting feature was the occurrence of 9 epidermoid carcinomata with a mean induction time of 469 ± 29 days and one basal cell carcinoma arising from irradiated skin. There were 12 glandular carcinomata with a mean induction time of 400 ± 48 days, probably arising from irradiated mammary tissue, which in rats extends down to the knees. 23 fibroadenomata with a mean induction time of 483 ± 35 days were found in the irradiated rats, but not all of these were in the irradiated areas. Three fibroadenomata were found in the 30 control animals. Besides the epidermoid and glandular carcinomata there were two instances of leukaemia, both in irradiated rats treated with growth hormone. These rats had greatly enlarged livers and spleens, myeloid cells were present in the peripheral circulation, and leukaemic deposits were found in the ovary, adrenal glands, orbit and salivary glands. Another irradiated rat treated with growth hormone had a carcinoma of the

lung. A lymphosarcoma was present in the thymus of two rats, and in another rat a greatly enlarged hyperplastic thymus was found.

A small adenoma of the pituitary was found in 4 rats. In Group A, one of these adenomata showed numerous mitoses.

Small adenocarcinomata of the thyroid were found in 4 rats. Two of these tumours were present in non-irradiated controls, which were over two years old.

DISCUSSION

A. *The dosage of radiation which will induce bone sarcomata*

The dosage of radiation which will induce bone sarcomata resolves into two quite different problems. First, the dosage required in the bone in terms of rad. This problem we have attempted to solve by the use of gamma radiations from a 192-Iridium source. It has been shown that 3000 r applied to both knee joints of the rat (equivalent to 4 major epiphyses and 4 cm. of bone shaft) have produced osteosarcomata in 34 of 116 rats (29·3 per cent), with a mean induction period of 488 ± 44 days.

The second problem concerns the applied dosage of 220 or 250 KVp. X-rays which would have a similar effect. On the basis of a differential absorption rate of bone for 220 KVp. X-rays of about 3 times the applied dose, it would be expected that 1000 r would be equally effective. In 123 irradiated rats at risk, Koletsky and Gustafson (1955) produced 5 osteogenic sarcomata by using 660 r of whole-body irradiation. This incidence is not strictly comparable to the incidence of osteosarcomata obtained in our experiments, because of the differences in the biological effects of whole-body and local irradiation. In the 39 cases of post-radiation bone sarcomata in man, collected by Cahan *et al.*, in 1948, the lowest dose was 1500 r. However, in discussing these cases, Vaughan (1956) noted that, except for one case following irradiation for fibrous dysplasia and a case of chondrosarcoma following irradiation for a bone cyst, the lowest dose used was 3000 r. Similarly, of the 13 cases reported by Sabanas *et al.* (1956) that had received only external radiation, the total dose was not known in two, it was 10,000 r in a case of preventive radiation for carcinoma of the breast, and in all the other cases the radiation treatment was preceded by either a bone cyst or a giant cell tumour, and the eventual bone sarcomata all arose at the sites of the previous lesions. Therefore if cases with an antecedent history of bone cyst, fibrous dysplasia or giant cell tumour are excluded, the lowest dose on record capable of inducing bone sarcomata in a previously normal bone is 3000 r X-rays. The induction period in Sabanas' group of cases varied between 32 months and 30 years (average more than 10 years), while in Cahan's group, it varied between 3 and 32 years, with a mean of 8·6 years.

The comparative dose in rads of bone-seeking radio-isotopes is difficult to calculate, but an estimate of the mean radiation dose received by the skeleton over a given period of time can be made, if the energy of the radiation emitted by the isotope, the fraction of the injected activity going to the skeleton and the effective half-life in the skeleton are known (Lamerton, 1958). By observing the tables prepared in this way by Lamerton, it appears that with 45-Calcium, 89 and 90-Strontium and 226-Radium, from 2000 to 3000 rads in one year are required to obtain an incidence of osteo-sarcomata comparable to the one obtained

in our results. The only exception appears to be 234-Plutonium, for which a dose of about 500 rads is required.

B. *The effect of stimulation of the bone by hormone treatment upon the incidence of tumours.*

The experiment was planned to show whether the stimulation of bone growth or the rate of bone turnover by hormone treatment would increase the incidence or shorten the induction period of radiation-induced bone tumours. The incidence of osteo-sarcomata in children, adolescents and in old people in association with Paget's disease would indicate that growth changes in the bone are an important factor in human bone sarcomata. Evans *et al.* (1948) demonstrated in their classic experiments the effects of growth hormone on bones, which result in stimulation of cartilage growth and in periosteal osteogenesis (Becks *et al.*, 1949). The tibias were longer and the epiphyseal cartilages were wider than in the controls, and all bones examined histologically showed lines of accretion under the periosteum. These authors, however, used large doses for periods of time up to 14 months.

On the other hand, thyroxine has been shown to produce maturation of bone, (Ray, Asling, Simpson and Evans, 1950; Simpson, Asling and Evans, 1950); this was revealed by the full development of the ossification centres, atrophy of the epiphyseal cartilage and closure on the epiphyseal side, while the calcium and nitrogen contents were decreased (Baisset, Doust-Blazy, Montastruc and Planel 1954). In man, Follis (1953) has shown that hyperthyroidism produces osteitis fibrosa, osteomalacia and osteoporosis, and Wilkins (1955) observed delayed maturation of bone in hyperthyroidism.

With regard to growth hormone our experiments have not given a statistically valid answer, though the data do suggest a trend toward an increased incidence of osteosarcomata in the growth hormone treated group. However, treatment with thyroxine shortened the induction period of osteosarcoma and this finding is statistically significant. Though our doses of growth hormone were much smaller than those used by Evans *et al.* (1948) they were sufficient to induce a small but significant increase of growth. However, in the rat, even in the female, growth never really ceases, as the proximal epiphyses of tibia, humerus and fibula, and the distal epiphyses of radius and ulna close normally at an average age of

EXPLANATION OF PLATES

FIG. 4.—Diagnostic X-ray showing an early osteosarcoma of left tibia 386 days after irradiation (thyroxine-treated rat).

FIG. 5.—X-ray of the same rat at post-mortem (436 days after irradiation).

FIG. 7.—X-ray at post-mortem 720 days after irradiation showing osteosarcomata of both femora (rat had been treated with growth hormone and then thyroxine). The tumour on the left side had been present for 51 days, the one on the right side for 9 days. The large, round shadow on the right side is due to a fibro-adenoma.

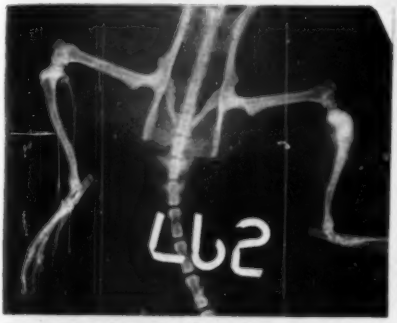
FIG. 8.—Photomicrograph of the osteosarcoma of the left femur illustrated in Fig. 7. ×2.

FIG. 9.—Photomicrograph illustrating the sharp boundary between the bone-forming (below) and the fleshy portions (above) of an osteosarcoma. (Irradiated rat treated with thryoxine, 283 days after irradiation, × 100).

FIG. 10.—Sclerosing osteosarcoma, metastasis in lung 489 days after irradiation (saline injected group D). × 100.

FIG. 11.—Fibrosarcoma invading muscle (irradiated rat treated with growth hormone; 452 days after irradiation). × 275. Note the striated muscle fibre.

FIG. 12.—Anaplastic sarcoma (irradiated rat treated with saline; 294 days after irradiation). × 275.



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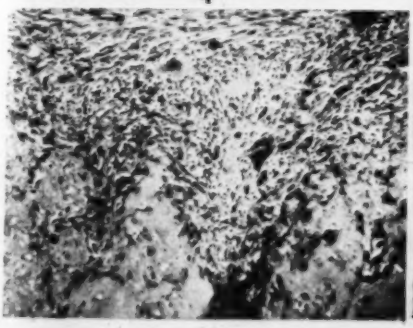

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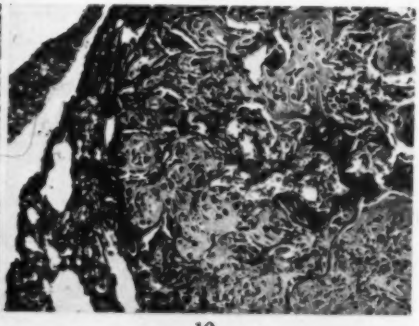

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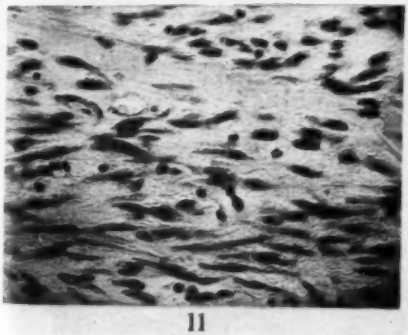

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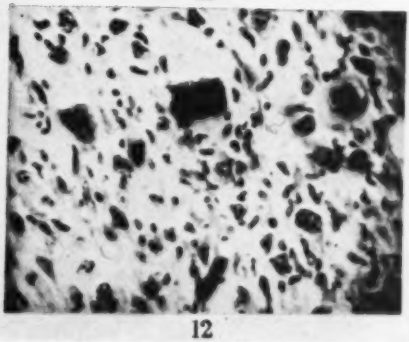

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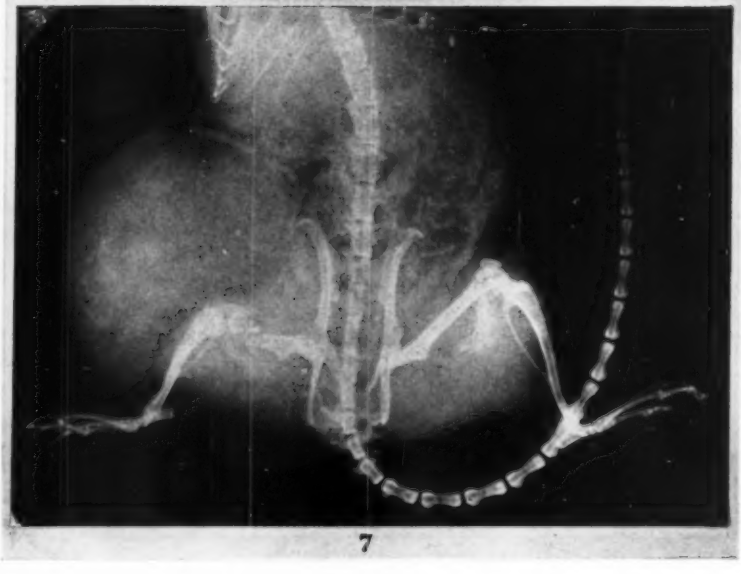

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Cater, Basergа and Lisco.

1135 days (Dawson, 1925), and it is possible that an extensive and prolonged alteration of the growth of bones must be induced before a striking effect can be demonstrated on the incidence of bone sarcomata. The rats were only 60 days old at the time of irradiation, therefore a marked proliferation of cells could be expected after the irradiation due to the natural growth of the bones. The growth of the irradiated bone was retarded compared with the unirradiated control, but some growth did take place and this might account for the considerable incidence of bone sarcomata in the irradiated group which was *not* treated with growth hormone.

Still, the incidence of osteosarcomata in growth hormone treated rats was somewhat higher than in controls, and the induction period in the thyroxine group was shorter, and in evaluating these results, one should not forget that these hormones did not have any appreciable effect on the incidence or induction period of the soft tissue sarcomata.

C. *The incidence of other neoplastic conditions*

In our experiments, 3000 r of gamma radiation induced 60 fibrosarcomata in 50 out of 116 rats. Though it is generally thought that post-irradiation sarcomata of the soft tissues are a rarity, experimental and clinical evidence support the view that this is not an infrequent complication of radiation. Warren (1943) has mentioned such a possibility, and Jones (1953) has reviewed the literature to which he added a case of his own, of soft tissue post-radiation sarcomata occurring in man. With 660 r of whole-body irradiation with X-rays, Koletsky and Gustafson (1955) produced 14 fibrosarcomata out of 123 irradiated rats at risk ; this incidence can be compared with 50 fibrosarcomata produced by 3000 r of gamma rays given locally in the present studies. Sarcomata of the soft tissues were also produced by Binhammer, Finerty, Schneider and Cunningham (1957) in single sublethally irradiated rats, and in parabiotic rats receiving 700 roentgens of X-rays.

The 12 cases of mammary adenocarcinomata produced in our experiments are not surprising, as radiation is known to increase the incidence of mammary carcinomata in rats. Actually, our incidence is low, when compared with the results obtained by Binhammer *et al.*, (1957) and by Shellabarger, Cronkite, Bond and Lippincott (1957) with whole-body irradiation, where the majority of the tumours produced were mammary carcinomata, but in whole-body irradiation the induced endocrine disturbance may be an important factor. The other tumours recorded in our experiments are not attributable to radiation, as their incidence was not significantly above that of non-irradiated rats. This applied also to the thyroid carcinomata, which have been shown (Lindsay, Potter and Chaikoff, 1957), to occur spontaneously in a high percentage of normal Long-Evans rats.

SUMMARY

1. A single dose of 3000 roentgen of gamma irradiation from a ^{192}Iridium teletherapy source applied to both knee joints of growing female rats induced osteosarcomata in 34 out of 116 rats. 50 rats developed sarcomata of soft tissues, 10 cancer of the skin, and 12 had mammary cancers.

2. 80 days after irradiation, ten-week courses of (A) growth hormone, (B) thyroxine (C) growth hormone followed by thyroxine and (D) saline were given

to study the effect of hormone treatment on the incidence and induction period of radiation-induced bone sarcomata.

3. 12 out of 30 growth hormone treated rats developed bone sarcomata compared with 7 out of 31 in the saline injected group.

4. Thyroxine treatment significantly reduced the mean latent period of radiation-induced osteosarcomata (521 $\pm$ 46 days in control Group D ; 388 $\pm$ 35 days in Group B).

5. The incidence of other types of malignant tumours was not affected by the hormone treatments.

We wish to thank Dr. T. B. Binns of Glaxo Laboratories Ltd., who kindly supplied Na 1 Thyroxine and Mr. F. Paulsen of Nordiska Hormon Laboratoriet Ab. Malmo, Sweden and the Wilson Laboratories of Chicago, U.S.A. for generous supplies of growth hormone.

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This investigation was begun while one of us (H.L.) was working in the Department of Radiotherapeutics of the University of Cambridge under a special grant from the British Empire Cancer Campaign which is gratefully acknowledged.

REFERENCES

Baisset, A., Douste-Blazy, L., Montastruc, P. and Planel, H.—(1954) *J. Physiol. Path. gén.*, **46**, 731.

Barr, J. S., Lingley, J. R. and Gall, E. A.—(1943) *Amer. J. Roentgenol.*, **49**, 104.

Becks, H., Asling, C. W., Simpson, M. E., Li, C. H. and Evans, H. M.—(1949) *Growth*, **13**, 175.

Binhammer, R. T., Finerty, J. C., Schneider, M. and Cunningham, A. W. B.—(1957) *Radiation Res.*, **6**, 339.

Cahan, W. G., Woodard, H. Q., Higinbotham, M. L., Stewart, F. W. and Coley, B. L.—(1948) *Cancer*, **1**, 3.

Dawson, A. B.—(1925) *Anat. Rec.*, **31**, 1.

Evans, H. M., Becks, H., Asling, C. W., Simpson, M. E. and Li, C. H.—(1948) *Growth*, **12**, 43.

Follis, R. H., Jr.—(1953) *Johns Hopk. Hosp. Bull.*, **92**, 405.

Freundlich, H. F. and Haybittle, J. L.—(1953) *Acta radiol., Stockh.*, **39**, 231.

Jones, A.—(1953) *Brit. J. Radiol.*, **26**, 273.

Koletsky, S. and Gustafson, G. E.—(1955) *Cancer Res.*, **15**, 100.

Lacassagne, A.—(1933) *C.R. Soc. Biol., Paris*, **112**, 562.

Idem and Vinzent, R.—(1929) *Ibid.*, **110**, 249.

Lamerton, L. F.—(1958) *Brit. J. Radiol.*, **31**, 229.

Lindsay, S., Potter, G. D. and Chaikoff, I. L.—(1957) *Cancer Res.*, **17**, 183.

Ludin, M.—(1930) *J. Radiol. Électrol.*, **14**, 255.

Marie, P., Clunet, J. and Roulot-Lapointe, G.—(1910) *Bull. Ass. franç. Cancer*, 3, 404.

Ray, R. D., Asling, C. W., Simpson, M. E. and Evans, H. M.—(1950) *Amer. J. Anat.*, **86**, 479.

SABANAS, A. O., DAHLIN, D. C., CHILDS, D. S. AND IVINS, J. C.—(1956) *Cancer*, **9**, 528.
SHELLABARGER, C. J., CRONKITE, E. P., BOND, V. P. AND LIPPINCOTT, S. W.—(1957) *Radiation Res.*, **6**, 501.
SIMPSON, M. E., ASLING, C. W. AND EVANS, H. M.—(1950) *Yale J. Biol. Med.*, **23**, 2.
SISSONS, H. A.—(1956) 'Progress in Radiobiology'. London (Oliver Boyd), p. 436.
SPIERS, F. W.—(1949) *Brit. J. Radiol.*, **22**, 521.
SUKHATME, P. V.—(1938) *Sankhyā*, **4**, 39.
VAUGHAN, J. M.—(1956) 'The Biochemistry and Physiology of Bone', ed. G. M. Bourne. New York (Academic Press), p. 729.
WARREN, S.—(1943) *Arch. Path.*, **35**, 340.
WILKINS, L.—(1955) *Ann. N.Y. Acad. Sci.*, **60**, 763.
WOODARD, H. Q. AND COLEY, B. L.—(1947) *Amer. J. Roentgenol.* **57**, 464.

SKIN GRAFTING IN THE STUDY OF COCARCINOGENESIS

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THE significance of the two-stage theory of carcinogenesis (e.g. Rous and Kidd, 1941 ; Berenblum, 1941, 1944 ; Mottram, 1944 ; Shubik, Baserga and Ritchie, 1953 ; Foulds, 1954) is widely recognised, although the range of species and tissues in which a two stage process has been demonstrated is narrow, and its mechanism is still far from being fully explained. We do not know, for instance, whether the essential changes which are manifested as " initiation " and " promotion " occur in the tissue in which tumours arise, or in some other part of the organism, nor whether the genetically determined differences in susceptibility to carcinogenesis are local or systemic characters.

An attempt has been made to gain some insight into these problems, in the case of skin carcinogenesis in mice.

Mouse skin after treatment with an initiating agent may appear both histologically and cytologically normal, e.g. at any time after one or more applications of urethane (Salaman and Roe, 1953), or may show only " occasional local epithelial thickening and a slight increase of keratin " e.g. 12 weeks after a single application of a carcinogenic hydrocarbon (Salaman and Gwynn, 1951), yet it behaves quite differently from untreated skin when subsequently treated with a promoting agent. It would be interesting to know whether this altered behaviour depends on a change in the skin itself, or in the rest of the animal, or in both. Orr and his collegues (Marchant and Orr, 1953) have brought forward evidence to show that the dermis plays an essential part in skin carcinogenesis, and hold the view that changes in the epidermis, the tissue from which the tumours eventually arise, are secondary. In the present work no attempt has been made to distinguish between the parts played by dermis and epidermis, but attention has been directed to the relation between the skin as a whole and the rest of the organism.

It is well-known that there are marked differences between different strains of mice in their susceptibility to carcinogenesis in various tissues (Bonser, 1940 ; Cowen, 1950 ; Salaman, 1959). In some cases, e.g. that of urethane-induced lung adenomas in the mouse (Schapiro and Kirschbaum, 1951) it seems clear that susceptibility resides in the tissue in which tumours arise and that the influence of the host was unimportant. The present work was undertaken to investigate skin carcinogenesis from a similar point of view ; to discover whether the susceptibility of the skin is a property of that tissue, or dependent on influences exerted by the rest of the animal.

The technique of whole-thickness skin-grafting was used in these studies.

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MATERIALS AND METHODS

Mice.—The mice used were of 101 and CBA strains, bred in this laboratory by brother-sister mating. The former are much more susceptible than the latter to the induction of skin tumours by the standard tumour-initiating and promoting techniques used in this laboratory (Salaman, 1959). F_1 hybrids from both reciprocal crosses between these strains were found to have a susceptibility to the development of skin tumours intermediate between that of the parent strains. In the present work 101 ♂ × CBA ♀ F_1 hybrids of both sexes were used. All experiments were done on approximately equal numbers of mice of both sexes with the exception of Control Test 1(c) where all the mice were CBA males. The mice were fed on a caked diet prepared according to the Rowett Institute formula (Thomson, 1936), with water *ad libitum*. All mice were vaccinated with sheep lymph when 6–8 weeks old as a precaution against ectromelia (Salaman and Tomlinson, 1957), and only positive reactors were used. At the beginning of the experiments the mice were 8–10 weeks old.

Chemicals and their application.—Initiating treatment: 0·2 ml. of a 0·15 per cent (w/v) solution of 9 : 10-dimethyl-1 : 2-benzanthracene. (DMBA) in acetone (AR) was applied from a graduated 1 ml. pipette to the skin of the back after the hair had been clipped. Further treatment was delayed for 3 weeks, so that no appreciable DMBA should contaminate the recipients of grafts.

Promoting treatment: 0·3 ml. of a 0·1 per cent (v/v) solution of croton oil in acetone was applied weekly for 18 weeks.

Papillomas were counted weekly (occasionally missing a week) and in most cases the count at the 19th week was taken as a measure of tumour incidence for purposes of comparison. Croton oil was obtained from Messrs. Stafford Allen & Sons Ltd., 20 Wharf Road, N.1.

DMBA was obtained from Messrs. Light & Co. Ltd.

Grafting technique.—The method was based on that of Prehn (1953). The mice to be grafted had the hair clipped from their backs. They were anaesthetised with ether, and secured with surgical strapping to a warm operating table. A strictly sterile technique was not observed, but all the instruments were boiled, and sutures were kept in spirit and rinsed in sterile Ringer before use. A midline incision was made in the skin of the back, extending from the nape of the neck to the root of the tail, its ends extended transversely to one side, and these extensions joined by a fourth incision along the lateral line of the flank. About half of the skin of the back was thus removed. In the later experiments a thin plastic sheet was used as a pattern, so that pieces of skin to be cross-grafted were as nearly as possible the same size and shape. The dimensions of this pattern are shown in Fig. 1. Bleeding points soon dried spontaneously. Raw areas were temporarily covered with sterile gauze soaked in warm sterile Ringer's solution. The graft was transferred to a petri dish containing a few sheets of filter paper moistered with Ringer's solution, epithelial surface down. The panniculus carnosus was partly peeled, partly scraped off in one piece with a blunt knife. (This procedure was personally suggested by Dr. Billingham, and was found to increase the " take " rate).

The grafts were sutured in position with cotton, using one suture at each corner, one or two at upper and lower margins, three or four in the mid-line, and two at the lateral margin. To use more than this number of stitches probably imperilled

the grafts, presumably because of interference with vascularisation from the margin.

The mice were grafted in pairs of the same sex. Pieces of skin were taken from one side of one mouse, rotated so that the direction of hair growth was reversed, and placed on a denuded area on the other side of the other mouse, and vice versa. This was done for two reasons; to help show the limits of the graft (in practice the fine linear scar gave a good indication of this) and to show with certainty, by the direction of hair growth, that the graft had "taken". A successful graft is shown in Fig. 2., 4 weeks after the operation, before re-clipping the hair for treatment.

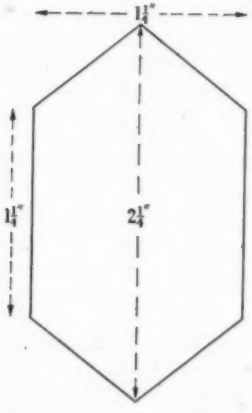

FIG. 1.—Either the right or left half of the pattern represents the area of skin removed from the back of the mouse.

No further dressing or treatment of any kind was found necessary. The mice were placed together (up to 8 in a box) after the operation, and by the second or third week almost all stitches had fallen out or had been nibbled away.

The mice were always left for four weeks, to allow thorough healing, before any substance was applied to the skin.

RESULTS

Although it has previously been shown that 101 and CBA mice are, respectively, susceptible and resistant to the development of skin tumours by initiation and promotion (Salaman, 1959), it was decided for the purpose of the present experiment to repeat the test on CBA mice and to show that grafting alone did not influence the response of these mice and their hybrids to initiation and promotion.

Control tests (a).—Thirteen male and thirteen female CBA mice were given the initiating application of DMBA and, after 3 weeks, 18 weekly applications of croton oil. The results are shown in Table I (*a*) together with comparable figures for the other strains as published elsewhere (Salaman, 1959).

(b). Twelve male and twelve female CBA mice were cross-grafted, and after waiting for four weeks for the grafts to become well established, they were given

the same initiating and promoting treatment. The results of these tests are shown in Table I (*b*). It is clear that CBAs are very much more resistant to this form of carcinogenesis than 101s, and that the operation of grafting does not materially alter their response. The number of tumours as shown were the maximum present at any time during the experiment.

(c). Twenty-four male 101 mice were cross-grafted. One month later they were given the initiating application of DMBA, and 3 weeks after that promotion with croton oil was started. The first tumours appeared after 4 weekly applications of croton oil, and increased in number until the end of the experiment, when all mice had tumours. Two mice did not survive the experiment and in another two the grafts did not take, these four mice are excluded. The results are recorded in Table I (*c*).

(d). Twelve male and 12 female 101 mice were given the initiating application of DMBA. Three weeks later all mice were cross-grafted in pairs. One male and 2 females died under the anaesthetic. All the grafts took. Four weeks after grafting, promoting treatment with croton oil was begun. Tumours started to appear on both the graft and the host skin after 3 weekly applications of croton oil, and increased in number thereafter till the end of the experiment. After 18 weeks of croton oil treatment, all mice had tumours. The results are recorded in Table I (*d*).

TABLE I.—*Results of Control Tests. Skin Tumour Incidence of Mice of Various Strains and the Effect on it of Isologous Skin Grafting. There were approximately Equal Numbers of Both Sexes in Each Group Except* (c), *Where all the Mice were Males.*

Test	Strain of mice	Number of mice	Number of tumours after 18 weeks of croton oil with the exception as shown: On host skin	On graft	On suture lines
(*a*)	CBA ungrafted	26	5	—	—
*	CBA ,,	20	0	After 12 weeks of croton oil	
*	101 ,,	35	390		
*	101 × CBA ungrafted	65	139		
(*b*)	CBA cross-grafted before initiation	20	3	0	0
(*c*)	101 ,, ,, ,,	20	206	64	19
(*d*)	101 cross-grafted after initiation	21	278	83	14

* From experiments described elsewhere (Salaman, 1959).

All the tumours referred to in this paper were papillomas, and were similar to those reported in previous studies in their appearance, and tendency to regress—particularly after croton oil was stopped. A number of sections made post mortem showed the usual histological appearances.

In this and later experiments, tumours which arose on the suture line and which could therefore neither be ascribed with certainty to the skin of the graft nor to that of the host mouse, were counted and recorded separately.

In tests (c) and (d), tumour incidence on the grafted part of the skin was appreciably less than on the host skin. The area of the graft was, in fact, slightly less

than half the treated area and it is unlikely that the operation of grafting either before or after the application of the initiator materially altered tumour incidence.

Experiment I

Grafting of initiated skin on to non-initiated mice and vice versa

Thirty-six 101 mice (eighteen males and eighteen females) were given the initiating application of DMBA. After three weeks they were cross-grafted with 36 untreated mice of the same strain and sex. Thus there were 36 DMBA-treated mice with untreated skin grafts and 36 untreated mice with DMBA-treated grafts. Four weeks after grafting all mice received croton oil weekly for 18 weeks.

Tumour incidence is recorded in Table II. Fig. 3 shows a DMBA-treated mouse grafted with untreated skin, after subsequent treatment with croton oil. The graft and tumours on the host skin and on the suture line, are visible.

TABLE II.—*Tumour Development in Skin Grafts and Host Skin after Cross-grafting Between "Initiated" and "Non-initiated" Isologous Mice, with Subsequent "Promotion".*

Number of cross-grafted 101 mice	Number of survivors with successful grafts	Number of tumours on: Host skin	Graft	Suture line
36 "initiated" mice	25	170	2	11
36 "non-initiated" mice	25	1	33	9

There were equal numbers of males and females in each group; cross-grafted pairs were of the same sex.

Experiment 2

Grafting of resistant skin on to susceptible mice

Twelve male and 12 female 101 × CBA hybrids had the skin on one half of their backs replaced by skin taken from CBAs. The CBA mice were sacrificed for this purpose and enough skin was obtained to graft the halves of two hybrids, whose skin was discarded. Otherwise the method was essentially the same as already described. All grafts took. After four weeks, the usual course of initiation and promotion was started. The results are shown in Table III (*a*) and represent the maximum numbers of tumours seen at any time during the experiment.

The experiment was repeated on 17 male and 15 female hybrids using much larger pieces of skin from the donor (CBA) mice, which covered nearly the whole of the backs. All grafts took. Further treatment was as before. Apart from one occasion when 2 extra transient tumours were seen on suture lines these numbers

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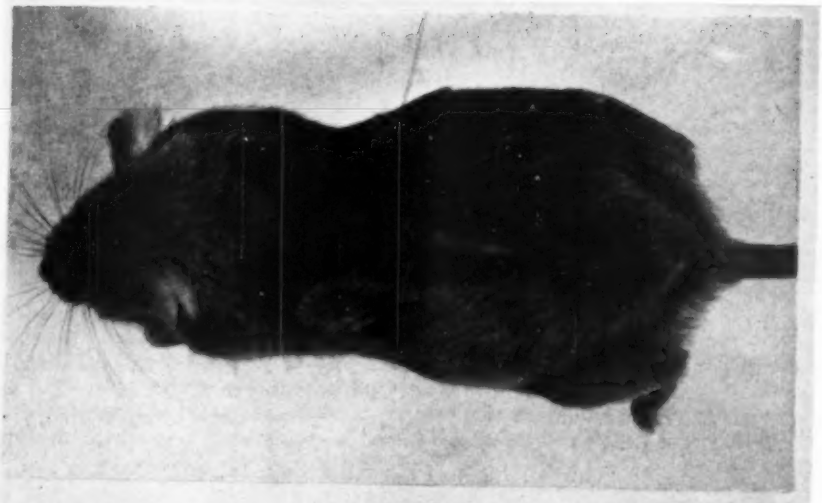
EXPLANATION OF PLATES.

FIG. 2.—A mouse four weeks after grafting by the method described.
Note reversed direction of hair growth on the graft.

FIG. 3.—DMBA-treated mouse grafted with untreated skin and then given croton oil. The graft is on the right—the caudal parts of the suture line and the reversed direction of hair growth are noticeable. Two tumours in the middle of the back are on the host skin, the third is on the suture line. Another tumour arising from the suture line is seen near the extreme caudal point of the graft.



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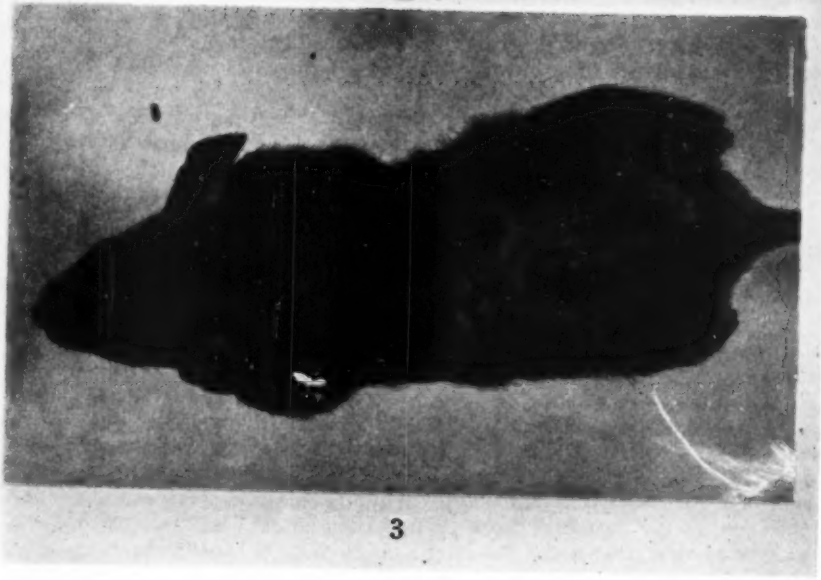

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represent the maximum number seen during the experiment. The results are shown in Table III (*b*).

TABLE III.—*Tumour Development in Skin from CBA (Resistant) Mice when Grafted to 101 × CBA Hybrids (Susceptible) after Subsequent "Initiation" and "Promotion".*

	Number of survivors with successful grafts	Number of tumours on: Host skin	Graft	Suture line
(*a*) 24 hybrids with half of back grafted	23	47	0	7
(*b*) 32 hybrids with whole of back grafted	32	7	0	1

Both sexes were approximately equally represented in each group.

It was apparent that grafts of skin of resistant mice which were transferred to susceptible mice remained resistant to the development of tumours.

No stitch abscesses, or other evidence of sepsis was ever seen at any time as a result of the operation of skin grafting.

No apparent sex difference in tumour response was noted in any of the experiments reported, and the results for both sexes have therefore been pooled.

DISCUSSION

It was early realized that differences between tumour incidence in different groups of treated mice, or between grafted and host skin, must be large if any conclusions were to be drawn from them. Previous experience made it improbable that small differences in average tumour counts would be statistically significant, because of individual variation. There were also unavoidable uncertainties connected with the method, e.g. possible stretching or shrinkage of grafts, and the difficulty of evaluating tumours which appeared on heads and limbs of host mice; areas not represented on the grafts. Fortunately the results were sufficiently clear-cut to allow certain definite deductions to be made.

The control series (Table I), shows the following:

(1) Susceptibility to skin tumour induction by initiation and promotion is much greater in 101 than in CBA mice.

(2) Susceptibility in 101 × CBA hybrids is intermediate between that of the parent strains.

(3) Isologous grafting of half the dorsal skin, either before initiation (in 101 and CBAs) or between initiation and promotion (in CBAs) does not appreciably alter tumour incidence (taking into consideration the fact that the final area of the graft was always slightly less than half the treated area of the back).

The results of isologous cross-grafting between "initiated" and "non-initiated" 101s (Experiment 1 and Table II) show that "initiation" is inherent in the skin, at any rate at 3 weeks after the application of the initiator. The normal host did not alter the tumour incidence on the "initiated" graft, nor did the normal graft alter that on the "initiated" host, when both were subsequently treated with the promoting agent. With the exception of a very few tumours which are liable to result from the action of croton oil alone, tumours arose only on initiated skin.

Exactly how the skin becomes initiated is not revealed by this work. Whether the DMBA acts directly on the epithelial cells or through a complex metabolic pathway is still unknown. Nevertheless we may say that by three weeks after initiation, the changes which produce the " latent cell " are complete, and the skin then behaves autonomously as far as its tumour producing propensities are concerned. It would be difficult to determine by grafting how soon after initiation with DMBA these changes occur, since the presence of excess DMBA on the treated skin might contaminate adjacent non-initiated skin if grafting were attempted too early. We can rule out contamination by the initiator in the present experiments since no tumours appeared on non-initiated skin.

The results of grafting resistant CBA skin on to susceptible 101 × CBA hybrids were also definite (Experiment 2 and Table III). Tumour incidence on the hybrid hosts was unaffected by the presence of the CBA grafts, and the grafts remained as insusceptible as they would have been if treated in their original position. Thus it appears that genetic differences in susceptibility to skin carcinogenesis by initiation and promotion depend on inherent properties of the skin itself.

For completeness, it would be desirable to graft susceptible skin on to a resistant host. Although hybrid mice are relatively more susceptible than their resistant parent, they are appreciably more resistant than their susceptible parent (Salaman, 1959). 101 skin could be grafted on to 101 × CBA hybrids, but it is unlikely that significant differences in incidence between grafted and host skin would be observed. It might also be possible to graft susceptible skin to a resistant mouse of a different strain after producing actively acquired tolerance of the foreign tissue (Billingham, Brent and Medawar, 1953 ; Billingham and Brent, 1956) by injecting cells of the donor strain into the future host mice *in utero* or when newborn. The method would be laborious because a low survival rate, and possibly a low success rate of grafting, would make it necessary to use very large numbers of animals.

A scrutiny of the results of the control experiments showed that the variation in tumour response between mice of the same strain (101) is due to environmental factors and not to inherent differences in the mice. The tumour yield of individual mice was studied and no correlation was found between the incidence of tumours on the mouse's own skin and the incidence on that part of it which was grafted on to another mouse. Neither was there a correlation between the incidence of tumours on the mouse's own skin and the incidence on the adjacent graft from another mouse. This is in keeping with the conclusion derived from genetic studies that the heterogeneity of response of individual mice of an inbred strain to carcinogenic treatment is due to environmental factors. Had the tumour response of each mouse depended on its genetic make-up, the correlations sought would have been found. The converse does not apply, for had these correlations been found the operation of environmental factors before grafting could not have been excluded.

Marchant and Orr (1953), on the basis of experiments in which isolated epidermal sheets were grafted, concluded that the action of carcinogens is primarily on the dermis, and that epidermal changes are secondary. The skin which was grafted in the experiments described here was of full thickness and certainly included the whole of the fibrous layer and part of the areolar layer of the dermis. Consequently the results are not relevant to the question considered by Orr and is colleagues. The conclusions reached, namely that (1) the condition of initiation

is localized in the skin and is transferred with it, and (2) genetically controlled susceptibility to carcinogenic treatment is also a local property of skin do not depend on the outcome of the controversy about the primary site of carcinogenic stimulation within the skin.

SUMMARY

1. Strain 101 mice were given a single "initiating" dose of DMBA to the skin of the back. Half the treated skin was then exchanged by grafting with that from untreated 101s. A course of croton oil applied to the whole skin of the back of all these mice elicited tumours only on skin (whether graft or host) which had been treated with DMBA.

2. Taking advantage of genetic differences in susceptibility to skin tumour induction between strains 101 and CBA, and their F1 hybrids, skin from CBA (resistant) mice was grafted on to 101 × CBA (susceptible) mice, which were then treated with DMBA and croton oil. The resistance of the grafted skin was not modified by transplantation to the susceptible host.

3. Control tests showed that the operation of grafting alone did not significantly affect skin tumour induction by DMBA followed by croton oil.

4. It was concluded that (a) the state of "initiation" and (*b*) the genetically controlled degree of susceptibility to skin tumour induction (by initiation followed by promotion), are properties inherent in the skin, since they are transferable by whole thickness grafting, and are unaffected by previous treatment or generic status of the host.

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REFERENCES

BERENBLUM, I.—(1941) *Cancer Res.*, **1**, 44.
Idem.—(1944) *Arch. Path.*, **38**, 233.
BILLINGHAM, R. E. AND BRENT, L.—(1956) *Proc. Roy. Soc.* B, **146**, 78.
Idem AND MEDAWAR, P. B.—(1953) *Nature*, **172**, 603.
BONSER, G. M.—(1940) *Amer. J. Cancer*, **38**, 319.
COWEN, P. N.—(1950) *Brit. J. Cancer*, **4**, 245.
FOULDS, L.—(1954) *Cancer Res.*, **14**, 327.
MARCHANT, J. AND ORR, J. W.—(1953) *Brit. J. Cancer*, **7**, 329.
MOTTRAM, J. C.—(1944) *J. Path. Bact.*, **56**, 391.
PREHN, R. T.—(1953) *J. nat. Cancer Inst.*, **13**, 859.
ROUS, P. AND KIDD, J. G.—(1941) *J. exp. Med.*, **73**, 365.
SALAMAN, M. H.—(1959) 'Carcinogenesis: Mechanisms of Action', Ciba Foundation. London (Churchill), p. 70.
Idem AND GWYNN, R. H.—(1951) *Brit. J. Cancer*, **5**, 252.
Idem AND ROE, F. J. C.—(1953) *Ibid.*, **7**, 472.
Idem AND TOMLINSON, A. J. H.—(1957) *J. Path. Bact.*, **74**, 17.
SHAPIRO, J. R. AND KIRSCHBAUM, A.—(1951) *Cancer Res.*, **8**, 644.
SHUBIK, P., BASERGA, R. AND RITCHIE, A. C.—(1953) *Brit. J. Cancer*, **7**, 342.
THOMSON, W.—(1936) *J. Hyg., Camb.*, **36**, 24.

INTRACELLULAR LOCALIZATION OF CARCINOGEN AND ITS RELATIONSHIP TO THE MECHANISM OF CARCINOGENESIS IN RAT LIVER

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THE experimental study of carcinogenesis aims at the finding of the common denominator for metabolic and structural changes which are characteristic of malignant transformation. In the previous paper (Fiala and Fiala, 1959) we tried to throw some light on this problem by correlating in time various changes during the carcinogenic process induced in rat liver by 3′-methyl, 4-dimethylaminoazobenzene. It was found that the whole process consists of two distinct phases. In the first there occurs a depletion of preformed cytoplasmic granules, accompanied by a lowering in the amount of respiratory activity, while glycolysis remains unchanged. Then, some 80 days after initiation of carcinogen feeding, a relatively sudden increase of anaerobic glycolysis takes place, coinciding with or slightly preceding the onset of massive cellular proliferation. At this time there occurs also a marked shift in the distribution of cytoplasmic ribonucleic acid from the ergastoplasm into the supernatant.

It is obvious, therefore, that the single factor we sought for is actually the ratio

$$\frac{N + S}{M + CH + R} = K^*$$

which changes its value in favor of the numerator in that critical period of carcinogenesis which marks the onset of malignant transformation. If we substitute the active masses of subcellular fractions for the respective symbols, the result describes the great preponderance of the nuclei and unorganized soluble phase of the cell over the preformed cytoplasmic elements. This amounts to the lack of cytoplasmic differentiation. If, on the other hand we substitute the corresponding ribonucleic acid for each fraction, we obtain a shift in the distribution of ribonucleic acid from cytoplasmic elements into the nucleus and supernatant. When, finally, we substitute into this ratio the metabolic activities of the corresponding fractions, we obtain a sudden increase in glycolysis (concentrated in the supernatant) over and above the respiration of mitochondria. The positive Warburg quotient

$$\frac{Q_{CO_2}^{O_2}}{Q_{O_2}} > 0$$

expressing the appearance of the aerobic glycolysis at this period is only an extreme case of this situation.

* *N* = nucleus.
M = mitochondria.
CH = "fluffy layer".
R = ergastoplasm.
S = soluble fraction.

If the process of carcinogenesis is conceived to be a shift from one equilibrium into another, then one or more factors must exist which keep this equilibrium within normal values. It seems plausible to assume that the protein-bound carcinogen interferes somehow with this regulating activity of the cell. In order to elucidate this fundamental problem of carcinogenesis, it is necessary to establish the localization of the carcinogen within the cell. It seems obvious that any subcellular organelle with a predilection for the carcinogen is most likely its primary target and plays an important role in this regulating activity of the cell. The questions arising next are, therefore, what kind of processes are influenced by the localization of the carcinogen and how is the interference by the carcinogen with the regulating activity of the cell, leading to a new equilibrium at the critical period of carcinogenesis, concerned with the mechanism of this process. These problems outline the program of this paper.

MATERIALS AND METHODS

Sprague-Dawley rats and, in a few experiments, Swiss albino mice were used as experimental animals. Separation and purification of subcellular fractions was performed as described in the previous paper.

Two criteria were used for estimating the purity of mitochondrial fractions: (a) lack of staining with fresh aqueous solution of Pyronin Y (0·1 per cent); (b) the basophilic quotient (B.Q.). The mitochondria apparently do not contain any ribonucleic acid (RNA) at all (Novikoff, 1956) or only small and variable amounts (Harel, Jacob and Moulé, 1957). Their B.Q. (RNA/protein nitrogen) should be, therefore, close to zero. On the other hand the B.Q. of the ergastoplasmic fraction approaches 1. For this reason the B.Q. of the mitochondrial fraction indicates approximately the percentage of contamination with basophilic RNA-rich elements. Thus a B.Q. of 0·5 indicates that the mitochondria are approximately 50 per cent pure, while a B.Q. of 0·2 indicates 80 per cent. purity.

The amount of protein-bound 3′-methyl, 4-dimethylaminoazobenzene (MDAB) was determined spectrophotometrically. The absorption of crystalline MDAB at pH 2·9 (absorption maximum at 520 mμ.) was used as a standard. The protein fraction was hydrolyzed, extracted with ether, the ethereal extracts evaporated to near dryness and residuum redissolved in acid ethanol (Miller and Miller, 1947).

Benzpyrene in 0·5 per cent benzene solution was painted twice on the hairless skin of 1–2 days old mice. The mice were sacrificed 24–48 hours later. The carcinogen was visible by characteristic fluorescence in long UV-light. The mice 1–2 days old were chosen because the skin of older mice is too difficult to homogenize. It is virtually impossible to separate their subcellular fractions because the broken tonofibrils adsorb indiscriminately all cytoplasmic elements. Even in young mice all cytoplasmic elements were heavily contaminated. The advantage of using very young mice for the study of carcinogen localization was confirmed recently by Calcutt (1958). The estimation of contamination of the mitochondrial fraction with ergastoplasm was based on the B.Q. The presence of the carcinogen was determined by measuring the fluorescence of the hydrolyzed protein residue of each fraction, previously precipitated with 5 per cent trichloroacetic acid (TCA) and extracted in a Soxhlet apparatus with boiling ethanol for 48 hours (Miller, 1951). The results were compared with unpainted controls of norma mice of the same litter. Fluorescence was measured using the fluorescent attachl-

ment of a Beckman DU spectrophotometer and taking the fluorescence of 5 mg. of quinine sulfate in 25 ml. water as standard equal to 100 per cent of the total deflection.

For the study of tryptophan peroxidase (TPO) L-tryptophan (78 mg. in 10 ml.) was injected intraperitoneally into half of the animals, while the other half remained uninjected. Six hours after injection all animals were sacrificed. TPO was assayed spectrophotometrically (Knox, 1955) from metaphosphoric acid (5 per cent) extracts of liver samples incubated at 37° for 1 hour and from unincubated controls. The sediments obtained after precipitation with 15 per cent metaphosphoric acid were extracted following Schneider's (1945) procedure and the deoxyribonucleic acid (DNA) was measured by the diphenylamine reaction. In the experiments concerning the effect of the carcinogen on TPO formation, single massive doses of carcinogen (40 mg. of crystalline MDAB dissolved in 10 ml. of corn oil) were injected intraperitoneally into rats kept on a basal diet for four weeks prior to the experiment and a similar number of control animals were injected with corn oil free of carcinogen. Seventeen hours after intraperitoneal injection one half of the control animals and one half of the carcinogen injected animals were injected with tryptophan.

Glucose 6-phosphatase (G 6-Pase) activity was determined by measuring the amount of liberated inorganic phosphate by King's modification of the Fiske-Subba-Row procedure (King, 1932). Homogenates or separated ergastoplasmic fractions were diluted appropriately with Tris-buffer of pH 7·0 and G 6-P (1 ml. of approx. 1×10^{-3} M sol.) was added when incubation was started (37°). After 15 minutes the reaction was stopped by adding an equal volume of 10 per cent TCA. In unincubated controls TCA was added before G 6-P. The total volume of mixture was 3·0 ml.

Polarographic determination of protein-bound sulphydryl groups was performed using Brdička ammoniacal solution of cobaltous chloride as indicated in the previous paper.

The proteins of the ergastoplasm were separated into soluble and insoluble fraction by the following procedure: the fraction spun down by 1 hour centrifugation at 59000 × g (Spinco preparative ultracentrifuge) from the homogenate in isotonic sucrose (10 volumes) was resuspended in cold physiological saline and recentrifuged in a Servall SS-1 centrifuge for 2 hours at 30000 × g in the cold room. The supernatant was discarded, the sediment was resuspended in distilled water, dialyzed against distilled water for 48 hours at 4° and lyophilized. The lyophilized material was homogenized at pH 9·2 either in a cold Edsall (1930) solution (6×10^{-1}KCl, 1×10^{-2}M Na_2CO_3, 4×10^{-2}M $NaHCO_3$) or, later, when it was seen that the reproducibility of the results demanded buffered solution, in Tris-buffer pH 9·1. The fraction was left for 1–2 hours in the cold with occasional stirring and then centrifuged for 1 hour at maximum speed in the Servall centrifuge. Both sediment, resuspended in distilled water, and supernatant were dialyzed against large volume of distilled water for 48 hours at 4°. The protein nitrogen of dialyzed subfractions was determined by Nesslerization.

RESULTS

1. *Localization of Carcinogen*

Measurements of isolated subcellular fractions from livers of rats fed with MDAB have shown that purified nuclei contained practically no carcinogen.

The nuclear fraction did not stain pink after precipitation with trichloracetic acid throughout the period of carcinogenesis if basophilic cytoplasmic contaminates were removed by repeated washings with isotonic sucrose. Also the hydrolysis of the purified nuclear fraction did not liberate any carcinogenic azo dye. This demonstrated that no appreciable amount of carcinogen was present in the nuclear fraction. The localization and action of this carcinogen must, therefore, be cytoplasmic.

The presence of the carcinogenic azo dye was noted in all cytoplasmic fractions after precipitation with 5 per cent TCA. The comparisons of amounts of MDAB liberated after hydrolysis of cytoplasmic fractions is shown in Table I. By far the greatest amount of this protein-bound carcinogen appeared in the soluble phase; considerably less was in the ergastoplasm; and very little was contained within the mitochondria. Still more significant was the fact that the purification of mitochondria, manifested in lowering of the B.Q. from 0·4 to 0·12 coincident with the removal of contaminating RNA-rich elements, resulted in a proportional loss of protein-bound carcinogen. The carcinogen content of liver mitochondria thus depended on contamination by ergastoplasm.

TABLE I.—*Distribution of MDAB in Cytoplasmic Fractions*

Fraction	Supernatant	Microsomes	Mitochondria I	Mitochondria II (Purified)
Protein nitrogen (mg.)	19·0	14	13	3
RNA (mg.)	11·4	15·4	5·2	0·36
B.Q.	0·6	1·1	0·4	0·12
Dye liberated (μg.)	70·0	46·0	16·0	1·1
Specific dye binding	3·62	3·3	1·2	0·36

Fig. 1 shows the absorption spectra of liberated azo dye from separated cytoplasmic fractions. The spectrum calculated for mitochondria with B.Q. approaching zero disappears and is, therefore, drawn as a straight line. Under the idealized conditions of pure mitochondria, no carcinogen would be found.

The slight content of the carcinogen in the mitochondrial fraction dependent on the contamination with ergastoplasm, made clear why this carcinogenic azo dye is not a respiratory inhibitor. This result warranted investigation of the localization of a carcinogenic fluorescent hydrocarbon such as benzopyrene and methylcholanthrene because it was speculated for a long time that mitochondria are the main storage place of these carcinogens. Thus Graffi (1939) treated various cells *in vitro* with benzopyrene and methylcholanthrene dissolved in glycerol and serum and observed by fluorescence their accumulation inside mitochondria stained by Janus Green. The obvious drawbacks of his observation were that he used *in vitro* experiments, which are often less conclusive than those *in vivo*, that he chose for his study cells in which benzopyrene and methylcholanthrene do not produce tumors.

In our experiments we have found that the location of benzopyrene in mouse skin depends on the mode of application. When the carcinogen was applied in lanoline, all cytoplasmic fractions yielded characteristic fluorescence in their alcoholic extracts while no carcinogen seemed to be liberated from protein residua after hydrolysis. On the other hand when the carcinogen was applied in benzene

solution, a significant fluorescence could be observed, even after a thorough alcohol extraction, from hydrolyzed protein residua of the supernatant and ergastoplasmic fractions. In the homogenate obtained from 2g. of mouse epidermis, the supernatant was the most strongly fluorescent; the nuclear fraction was least, although its nitrogen content was relatively the highest. The mitochondrial fraction formed only a comparatively small portion of the protein nitrogen of the homogenate. Its content of fluorescent material was also low after hydrolysis, but, per mg. of nitrogen it was only slightly lower than other cytoplasmic fractions.

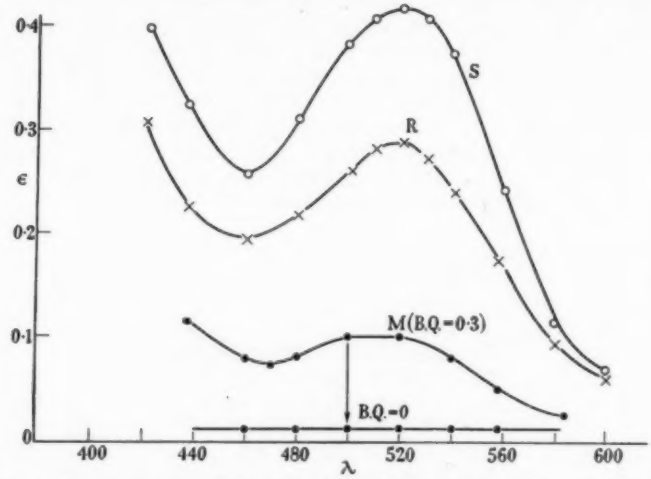

FIG. 1.—Protein-bound carcinogen liberated by hydrolysis from separated cytoplasmic fractions of 3 rat livers (43 days of carcinogen feeding). Ethanol-HCl mixture, Vol. 25 ml.

When we obtained in another experiment, a mitochondrial fraction which was less contaminated with RNA-rich elements, no fluorescence was found after hydrolysis.

These results left little doubt that neither MDAB nor 3,4-benzopyrene are stored in mitochondria, which is in conformity with their failure to produce respiratory inhibition. The mitochondria cannot be thought of as targets for any direct action of these carcinogens.

2. *Distribution of MDAB in ribonucleic acid-containing fractions of the cytoplasm*

The presence of protein-bound carcinogen in the supernatant and ergastoplasm, fractions containing ribonucleoproteins, and the absence of the carcinogen in mitochondria, free of RNA in any appreciable amount, necessitated investigation of the relationship of the carcinogen to nucleoproteins. When liver homogenate from rats fed with carcinogenic azo dye (28 days) was centrifuged at 164000 × g (0·88 M sucrose) for 4 hours, about 95 per cent of all ribonucleoprotein material was spun down. The protein-bound azo dye was, nevertheless, present in the supernatant in a greater amount than in all other fractions combined.*

* This experiment was performed (in the Spring of 1955) with the co-operation of Dr. K. McCarty of the Department of Biochemistry, Columbia University.

Attempts were also made to isolate, or at least to concentrate, the carcinogen-binding protein from the soluble phase. The initial steps of separation are shown in Table II. Practically all ribonucleoprotein was precipitated by acidification to pH 5·6. The fraction precipitated by the second addition of ammonium sulfate contained most of the protein-bound carcinogen but gave a completely negative pentose test (Mejbaum's modification of Bial's reaction).

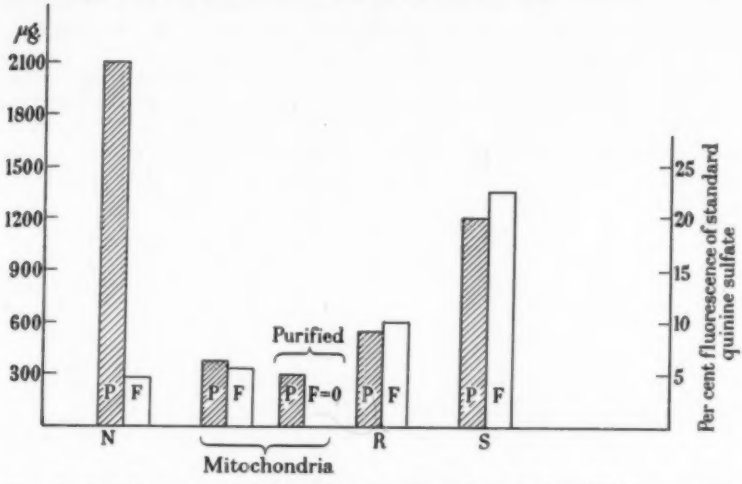

FIG. 2.—Relative proportions of protein nitrogen and protein-bound 3,4-benzpyrene in fractions of mouse epidermis.

TABLE II.—*Initial Steps of Purification of the Protein Binding the Azo Dye*

1. Livers removed separately and homogenized in cold 0·25 M sucrose.
2. Centrifuged, 10 minutes at 13,000 r.p.m. Sediment discarded.
3. Supernatant, 1 M. CH_3COOH added to pH 5·8. Centrifuged, sediment discarded.
4. Supernatant, 1N NH_4OH added to pH 8·0 + $(NH_4)_2SO4$ in powder to concentration 2M. Centrifuged, sediment discarded.
5. Supernatant + 0·1 M. CH_3COOH added to pH 5·4; if sediment forms within 10 minutes, centrifuged and sediment discarded.
6. Supernatant + $(NH_4)_2SO_4$ to 3 Moles, centrifuged, reddish supernatant discarded.
7.* Sediment dissolved in

$$\frac{N}{1000} - \frac{N}{100}$$

NH_4OH in a small volume, dialyzed against H_2O 48 hours.

8. Crude fraction lyophilized and stored under vacuum in the cold room.

This lyophilized fraction is the starting point for isolation of carcinogen-binding protein.
* 7 gives a negative pentose test.

The isolated protein fraction had a pale yellow color in solution. Its absorption maximum occurred at 405 mμ. (pH 7·0). The absorption maximum is shifted only slightly toward longer wave-lengths in comparison to the absorption maxi-

mum of pure carcinogen dissolved in diluted ethanol and at the same pH (Fig. 3). Normal hydrochloric acid, causing pink coloration, dissolved the protein-dye complex without causing turbidity. It was seen that under these conditions the absorption maximum of the complex was again similar to that of pure carcinogen.

In the ultraviolet region the undenatured protein fraction containing very low absorption at 260 mμ. had an absorption peak at 280 mμ. The negative pentose test and spectra thus presented convincing evidence that carcinogen-binding protein of the supernatant fraction is not a ribonucleoprotein.

In order to establish the relationship of the carcinogen to the ribonucleoproteins in the ergastoplasm, the lyophilized subfractions (pH 9·1 soluble sub-

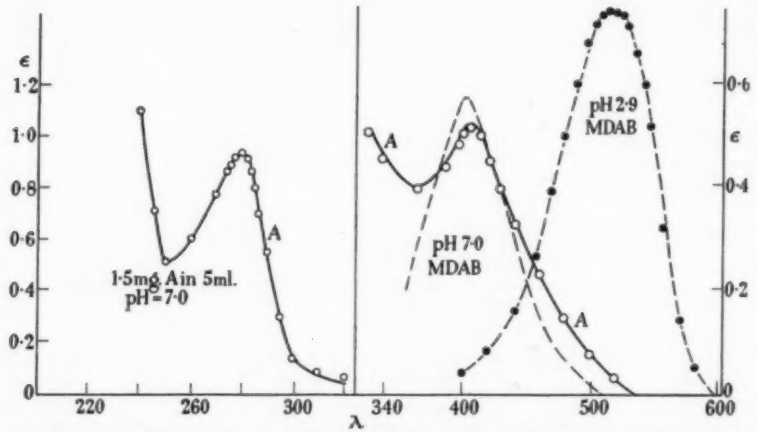

FIG. 3.—Absorption spectra of carcinogen-binding protein (full line) in ultraviolet and visible region. Spectra of crystalline carcinogen (10 μg./ml.) at pH 2·9 and 7·0.

fraction and the residuum) were analyzed for ribonucleic acid. It was found that the soluble portion contained practically all the ribonucleoprotein which could be precipitated with $CaCl_2$ and also a protein fraction which did not contain RNA but was relatively rich in polarographically active, protein-bound, sulphydryl groups. The RNA-rich protein fraction could be split off from the soluble subfraction with 1 M NaCl.

The pH 9·1-insoluble residuum dissolved in N/2 NaOH at room temperature only in the course of several hours, giving a yellow solution. Approximately 1/3 of this residuum dissolved in 0·1 M citric acid (pH 2·5), while the addition of 0·1 M Na citrate produced turbidity. In contrast to the soluble fraction, the insoluble residuum was characterized by a low content of polarographically active sulphydryl groups.

The amount of sulphydryl groups in the residuum did not increase after heating to 60° for 15 minutes to any considerable degree. It seemed likely that their presence in the residuum was due to the contamination by the soluble fraction. The residuum differed also from the soluble fraction by a relatively low absorption at 280 mμ. which belongs to aromatic amino acids.

After several hours, the yellow material of the ergastoplasmic residuum dissolved in acidified chloroform. When irradiated in the dark with long ultraviolet light it gave a very intensive green fluorescence. This indicated that the riboflavin (lumiflavin) moiety of yellow enzymes is incorporated into the ergastoplasmic residuum. Ergastoplasmic residuum from tumors such as Novikoff hepatoma did not yield fluorescence under similar conditions. The properties of the residuum and the fact that it contains few if any sulphydryl groups and is very poor in aromatic amino acids, left no doubt that the residuum represents the insoluble, structural, protein framework of the ergastoplasm, while the RNA-rich material represents Palade granules (Palade, 1955) which are attached from the outside to the tubular network (endoplasmic reticulum, Porter, 1953).

Because of the solubility of carcinogen-binding protein in the supernatant, we expected to find carcinogen localized in the soluble subfraction of the ergasto-

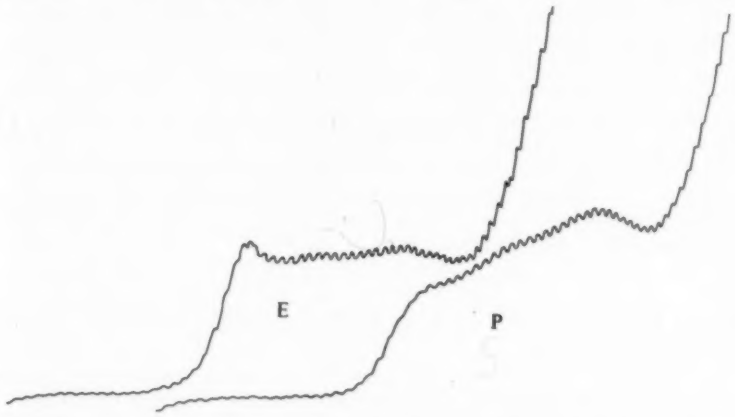

FIG. 4.—Comparison of polarographically active free sulphydryl groups in structural (E) and soluble (P) protein fraction of rat liver ergastoplasm.

13 mg. of lyophilized protein fraction dissolved in 5 ml. of 0·5 N NaOH. 0·5 ml. of protein solution added to the mixture of 5 ml. N/10 NH_4Cl in N/10 NH_4OH and 0·5 ml. Co $(NH_3)_6Cl_3$ (4×10^{-3} M).

plasm. Contrary to this expectation the carcinogen was found to be firmly bound mainly to the residuum, apart from ribonucleoproteins, thus providing evidence that it is here where the damaging influence of this carcinogen is primarily localized.

3. *The prevention of adaptive formation of tryptophan peroxidase by MDAB*

All changes described so far as induced by carcinogens are long range effects. By the fact that carcinogenic azo dye MDAB is not a respiratory inhibitor and that glycolysis increases only at the onset of malignant transformation (Fiala and Fiala, 1959) the often held assumption that the carcinogen interferes primarily with the metabolism of the cell cannot be held any longer. What then is the primary effect of a carcinogen beyond its binding to one or more chemically, not yet fully characterized proteins ?

It seemed obvious that an answer to this question could be obtained if we could find a reaction normally occurring either in the supernatant or in the ergastoplasm which would be inhibited by a carcinogen very soon after its incorporation into the cell. We assumed that the depletion of cytoplasmic structures may be due to the interference with some phase of the mechanism of protein synthesis. This possibility could not be excluded by the finding that carcinogen-binding protein is not a nucleoprotein, because the mechanism of protein synthesis is very likely caused by an interplay of several components, as one may judge, e.g. from the experiments of Hoagland, Keller and Zamecnik (1956). One of the best objects in the study of protein synthesis are the adaptive enzymes. It is known that tryptophan peroxidase (TPO) is an adaptive enzyme formed in mammalian liver in increased amounts following the injection of L-tryptophan (Knox, 1951). The enzyme is synthesized *de novo* from its constituent amino acids (Lee and Williams, 1952). Moreover, it has been reported recently (Claudatus and Ginori, 1957) that there exists a block in the pathway of tryptophan metabolism in hepatoma via kynurenin, indicating that TPO is absent from hepatoma. When we tested the localization of this enzyme in normal rat liver, we found that it is present in the soluble phase. No data seem to exist in the literature on the level of this enzyme and its adaptive synthesis in the precancerous liver.

For those reasons we investigated the induction of TPO in livers of MDAB-fed rats and compared it with controls on a basal diet without carcinogen. We found that the level of TPO did not appreciably change during the first stage of carcinogenesis but that its new formation was largely inhibited by the presence of carcinogen. This is shown in Table III.

TABLE III.—*Effect of MDAB Feeding on Adaptive Synthesis of Tryptophan Peroxidase*

			Time in days					
			0	10	25	30	35	80
Basal diet	Non-injected	Activity	0·420	0·724	0·484	0·287	0·429	0·640
		DNA	702	740	570	544	700	576
		Spec. act.	0·300	0·489	0·368	0·263	0·300	0·556
	Tryptophan injected	Activity	1·550	1·580	1·240	1·130	1·410	1·672
		DNA	786	552	500	584	700	524
		Spec. act.	0·987	1·436	1·240	0·966	1·007	1·024
		Increase	0·687	0·947	0·882	0·703	0·707	1·024
			229%	195%	240%	168%	230%	184%
Basal diet plus carcinogen	Non-injected	Activity	—	0·620	0·384	0·366	0·420	0·440
		DNA	—	750	530	764	500	760
		Spec. act.	—	0·413	0·359	0·476	0·420	0·290
	Tryptophan injected	Activity	—	1·130	0·480	0·476	0·829	0·550
		DNA	—	675	530	572	700	692
		Spec. act.	—	0·836	0·452	0·417	0·440	0·410
		Increase	—	0·423	0·093	0	0·020	0·120
				100%	25%	—	—	40%

Activity = micro-moles of Kynurenin formed in 1 ml. homogenate in 1 hour at 37°.
DNA = μg. in 1 ml. homogenate.
Spec. act. = activity per 5×10^{-12} g. DNA.

From these data one can conclude that the carcinogen does indeed inhibit the synthesis of at least some soluble proteins.

In order to decide whether this inhibition is caused by structural damage to the cell, which certainly occurs already during first weeks of carcinogen feeding, or whether it is due to a block of the enzyme forming system by the carcinogen, we applied single massive doses of carcinogen, followed by tryptophan injection and measured TPO activity some six hours later. The whole experiment was performed within twenty-four hours after application of the carcinogen. Table IV shows the result of such experiment.

TABLE IV.—*Effect of a Single Injection of MDAB on Adaptive Synthesis of Tryptophan Peroxidase*

Corn oil	Non-injected (3)	Activity	0·600		
		DNA	744		
		Spec. act.	0·400		
	Injected (Trypt.) (3)	Activity	1·300		
		DNA	608		
		Spec. act.	1·060		
		Increase	0·660, 165%		
Corn oil plus carcinogen	Non-injected (3)	Activity	0·310	0·550	0·435
		DNA	615	552	550
		Spec. act.	0·258	0·500	0·395
	Injected (3)	Activity	0·615	0·810	0·303
		DNA	600	608	450
		Spec. act.	0·512	0·670	0·330
		Increase	0·254 100%	0·170	0

It is seen that there is a substantial inhibition of TPO formation already after a single injection of the carcinogen so that we may assume with a great degree of probability that it is not structural damage but a block by the carcinogen which is responsible for the inhibition of protein synthesis by the enzyme forming system of the cell.

4. *The inhibiting effect of the carcinogen on the ergastoplasm*

Since the carcinogenic azo dye MDAB is present not only in the soluble phase of the cell, but also in the residuum of the ergastoplasm, the question of how the residuum is affected by the carcinogen led to a problem similar to the one encountered in the case of TPO and the soluble phase of the cell, namely to the choice of a suitable enzyme. Such an enzyme is G 6-Pase, shown to be present exclusively in the microsomes (de Duve *et al.*, 1953). This enzyme was shown to decline in amount during hepatic carcinogenesis and to be absent from hepatoma (Weber and Cantero, 1955). It could be assumed, therefore, that an investigation of the mechanism whereby the carcinogen brings about the depletion of G 6-Pase, would throw some light on the effect of the carcinogen on the ergastoplasm.

The separation of the ergastoplasm into pH 9·1-soluble fraction and the insoluble residuum has shown that this enzyme is present exclusively in the residuum. This agrees with the unsuccessful attempts to solubilize the enzyme.

When we followed the depletion of G 6-Pase during feeding with MDAB we

have seen that for a period of several weeks or 2–3 months there is no appreciable change in the level of this enzyme. This agrees with the data of Weber and Cantero (1955) who used DAB as carcinogen. When we correlated the level of this enzyme with the level of anaerobic glycolysis and cellular proliferation we have seen that the period of depletion of G 6-Pase roughly coincides with the sudden increase of anaerobic glycolysis and, within certain limits of error, also with the onset of cellular proliferation. The coincidence of G 6-Pase depletion and of anaerobic glycolysis increase seems to be an important point, which is documented by Table V.

TABLE V.—*Time-Correlation between G 6-Pase, Increase of Anaerobic Glycolysis and Cellular Proliferation during MDAB Carcinogenesis*

Days	G 6-Pase activity	Anaerobic glycolysis increase in %	DNA content of whole liver in 10^6 units (5×10^{-12} g. DNA)	Proliferation
32	100	0	3200	0
40	100	0	3450	0
55	100	0	2800	0
60	100	0	3100	0
75	50	†40	4400	†
76	42	†200	8000	†††
87	80	0	3900	0
105	48	†55	4250	†
150	25	†195	5200	††

In further experiments we have seen that single massive doses may decrease the activity of G 6-Pase. However, this effect did not occur regularly and we did not attempt, so far, to further analyze this phenomenon. It seemed more important to study whether the increase in the level of G 6-Pase observed by Weber, Allard, Lamirande and Cantero (1955) induced in the normal liver by several injections of cortisone acetate does occur in the carcinogen storing liver. Under these conditions the increase of G 6-Pase seems to increase as an adaptive enzyme. In rats fed with a basal diet and, therefore, under a low protein regimen, the cortisone injection often led to a striking decrease in the weight of liver, instead of increase in the weight usually observed in the normal rats. Such cases were excluded and only these cases were considered in which liver either did not change or increased its weight after 5 consecutive daily injections of 25 mg. of cortisone acetate. This was achieved by placing the animals on regular food during the whole period of experiment. In such cases the increase of G 6-Pase occurred in control, cortisone-injected, animals, but did not occur in carcinogen-treated, cortisone-injected, rats. In order to see whether one or two injections of the carcinogen in normal rats would modify the response of tissue to cortisone in regard to G 6-Pase, the following experiment illustrated in Table VI was performed.

The experiment shows that similar to the effect on TPO in the soluble phase, the carcinogen suppresses the increase of an adaptive enzyme of ergastoplasm. The condition, however, may not be strictly analogous since a decrease in G 6-Pase activity was occasionally observed, after single massive doses of carcinogen in distinction to the TPO level which remained unchanged. In any case the carcinogen also exerts an inhibitory effect on the ergastoplasm which is manifested long before structural damage can be observed by differential centrifugation.

TABLE VI.—*Effect of Cortisone Acetate on the Activity of G 6-Pase of Rat Liver in the Presence of MDAB*

	Cortisone	A (8) (μg.)	B (8) (μg.)	C (8) (μg.)
Controls	Non-injected	10·6	9·87	7·81
	Injected	23·4	18·44	11·65
Increase	—	+120%	+87%	+50%
Carcinogen	Non-injected	10·4	9·3	4·15*
	Injected	12·66	11·23	7·77†
Increase	—	+22%	+20%	+ ?

A = 2 injections of corn oil (10 ml.) with or without carcinogen (60 mg.).
B = Basal diet to 60 days, with or without carcinogen.
C = Basal diet for 100 days, with or without carcinogen.
Note.—Activity = μg. inorganic P released in 20 minutes at 37° by tissue homogenate equivalent to 0·05 mg. DNA.
* Malignant changes (gross proliferation) in both livers.
† i.d. in one liver.

5. *The effect of MDAB on the structure of the ergastoplasm*

In the critical stage of liver carcinogenesis the sudden increase of anaerobic glycolysis is linked to the depletion of G 6-Pase. This enzyme has been found (see above) to be anchored in the insoluble residuum of the ergastoplasm while the ribonucleoprotein fraction goes into solution at pH 9·1. This fact led us to investigate what happens to the insoluble residuum at this stage of carcinogenesis and we compared, therefore, the quantitative relationship between the residuum and the soluble fraction during carcinogenesis. The results are summarized in Table VII.

TABLE VII.—*The Proportion between Insoluble (pH 9·0) Residuum (I) and Soluble Proteins (S) in the Ergastoplasm Fraction during Rat Liver Carcinogenesis*

	Basal diet		MDAB						Novikoff hepatoma
Time (days)	43	105	43	70	87	100	105	119	—
I/S	2·0	1·9	2·0	2·0	1·4	0·66	0·55	0·50	0·4
Total DNA of liver*	2709	3760	3600	3250	3300	4000	4690	5100.	—
Gross proliferation	−	−	−	−	−	±	+	+	+

* Expressed in 10^6 DNA units (5×10^{-12} g.).

The table shows that the proportion between the residuum and the soluble fraction does not undergo a noticeable change in the first stage of carcinogenesis. In the second period, however, there occurs a striking change. The same amount of lyophilized material in the control shows a predominance of the residuum, while in the experimental sample and especially in tumor (Novikoff hepatoma) the ratio of the residuum to the soluble fraction is reversed, demonstrating predominance of the soluble fraction and depletion of the residuum, which is the structural groundwork of the ergastoplasm.

DISCUSSION

The metabolic damage to the cell induced by a carcinogen such as MDAB is undoubtedly a secondary, long-range effect; a result of whole series of events

which are still largely unknown. A more immediate effect than the damage to respiration, is an inhibition of synthesis of certain proteins. The case of the inhibition of TPO formation is an example which is suggestive of harm done by the carcinogen to the ability of the cell to adapt itself to the demands of the organism. At the same time this loss may mean an initial step in progressively increasing independence of the cell from the influence of the environment. This probably represents from a biological viewpoint a gain for the cell which exists on a relatively small rate of protein synthesis when on a basal diet, and is further encroached upon by an additional decrease in protein synthesis in the presence of carcinogen. It has been shown (Westerfeld, Rickert and Hilfinger, 1950) that e.g. xanthine oxidase is considerably depleted in livers of rats on a basal diet and that the carcinogen only accentuates this depletion. Something similar occurs with the levels of respiratory enzymes. In the presence of the carcinogen, the cell becomes increasingly independent of the organism; becoming so to speak a tissue culture within the organism.

From this viewpoint it is probably of utmost importance that the carcinogen is incorporated into the insoluble residuum which constitutes the tubular structures of the ergastoplasm. The system of these structures is known to form a continuous network of membrane-bound cavities, called endoplasmic reticulum (Porter, 1953, Palade, 1955). In distinction to the soluble proteins of the cell, endowed with various enzymatic functions, the insoluble proteins were often considered of secondary importance, merely acting in the role of a cytoskeleton (Needham, 1936, Peters, 1936). It seems, however, more likely that the structural proteins of the cell have a role of utmost importance. Ruska, Edwards and Caesar, (1957) suggested that the tubular network of the endoplasmic reticulum is the conductor of intracellular information and connects the nucleus with the cytoplasm in one system of membranes, including the external cellular membrane. Our experiments have shown that in the critical stage of carcinogenesis there occurs a depletion of this structural protein system of ergastoplasm. It seems very likely that the differences observed in membranes of tumor mitochondria (Mutolo and Abrignani, 1957) are another manifestation of this damage to the structural membrane network of the cell.

For this concept, the fact that sudden changes along several parameters (distribution of sulfhydryl groups, cytoplasmic ribonucleic acid, increase of glycolysis, massive proliferation) are coincident with the substantial loss of structural protein from the ergastoplasm is of great importance for the following reasons: It seems plausible to assume that the residual protein of the ergastoplasm is not only the receptor for carcinogen, as we have shown in case of MDAB, but also for tropic hormones. This would agree with the observation that ergastoplasm is the main target for hormones such as ACTH, FSH and TSH (Fiala, Fiala and Sproul, 1957; Fiala, Sproul and Fiala, 1956, 1957). As far as we can judge from early studies of the effect of sex hormones on the epithelial cells of seminal vesicles in the rat (Moore, Hughes and Gallagher, 1930), these hormonal effects are analogous to those exerted by anterior pituitary hormones. It seems, therefore, that findings pointing to ergastoplasm as the main hormonal target are probably true and in agreement with the concept of Ruska, Edwards and Caesar (1957) concerning the role of the tubular network of the ergastoplasm for the cell: The sudden throwing away of the structural protein would be an adaptive reaction for the cell, allowing it to escape its social duties to the organism by not maintaining synthesis of many

proteins, non-essential to its survival, but formed because of essential functions. By throwing this burden away, the cell solves its problem of adaptation.

From this viewpoint, several observations are easily understood. The loss of structural protein from the ergastoplasm means disintegration of the tubular network. The RNA-rich granules (Palade-granules) which are attached to the outer surface of these tubules (Palade 1955), are no longer held in an orderly arrangement or in quantitatively sufficient amounts by these tubules. The Palade granules, now randomly distributed, may either disappear or become independent and present in increased amounts within the soluble phase. This explains the observed redistribution of cytoplasmic RNA, reported in the previous paper. Since G 6-Pase is anchored to the residuum, its loss in the critical stage of carcinogenesis helps in increasing the concentration of glycolytic intermediates. An analogous circumstance seems to be valid for hexosodiphosphatase, indicating that the conditions for increased glycolysis may again be explained by the loss of ergastoplasmic residuum. This represents an additional gain for the cell whose mitochondria became scarce.

On the basis of the circumstance that both hormones and carcinogen act on the same structural component and following the concept (Fiala, 1958) that considers the cell as a homeostat, one can understand easily why a hormone may exert, under certain conditions, a carcinogenic effect, as is known for oestrogen (Lacassagne, 1933). The negative feedback in a homeostatic mechanism can easily become positive. It may not be too far fetched to consider the action of certain carcinogenic compounds as an action of " false hormones ". In any case one can draw from our experiments the conclusion that the localization of foreign compounds at a vitally important position within, the cell, that is the structural protein of the ergastoplasm, and its ejection by the cell, results in malignant transformation of the cell.

SUMMARY

The experimental evidence was given to show that carcinogenic azo dye 3′-methyl, 4-dimethylaminoazobenzene is localized in the ergastoplasm and the soluble phase of the cell. The same is true for 3,4 benzopyrene in mouse epidermis. The mitochondria do not contain protein-bound carcinogen. This substantiates the fact that these compounds are not respiratory inhibitors.

The carcinogen-binding proteins in the ergastoplasm and in the soluble phase are distinct from nucleoproteins. The ergastoplasm was fractionated into an insoluble residuum, poor in sulphydryl groups and in aromatic aminoacids, and into a soluble fraction. The former contains the carcinogen, the latter ribonucleic acid.

The protein-bound carcinogen inhibits new formation of an adaptive liver enzyme, tryptophan peroxidase, and also inhibits the cortisone induced increase of glucose-6-phosphatase even after a single application.

The insoluble residuum contains glucose-6-phosphatase. Its disappearance from the cell coincides with increased anaerobic glycolysis.

This stage in carcinogenesis is marked by the depletion of the residual structural protein from the ergastoplasm.

It was concluded that binding of the carcinogen to this intracellular locus and its consequent irreversible loss means the liberation of the cell from its social

duties to the organism, this being identical with the malignant transformation of the cell.

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REFERENCES

CALCUTT, G.—(1958) *Brit. J. Cancer*, **12**, 149.
CLAUDATUS, J. AND GINORI, S.—(1957) *Science*, **125**, 394.
DE DUVE, C., GIANETTO, R., APPELMANS, F. AND WATTIAUX, R.—(1953) *Nature, Lond.*, **172**, 1143.
EDSALL, J. T.—(1930) *J. biol. Chem.*, **89**, 289.
FIALA, S.—(1958) *IV Int. Congr. Biochem., Vienna.* Abstract, Section 6, 74.
Idem AND FIALA, A. E.—(1959) *Brit. J. Cancer*, **13**, 136.
Idem, FIALA, A. E. AND SPROUL, E. E.—(1957) *Proc. Amer. Ass. Cancer Res.*, **2**, 200.
Idem, SPROUL, E. E. AND FIALA, A. E.—(1956) *J. biophys. biochem. Cytol.*, **2**, 115.—(1957) *Proc. Soc. exp. Biol.*, N.Y., **94**, 517.
GRAFFI, A.—(1939) *Z. Krebsforsch.*, **49**, 477.
HAREL, L., JACOB, A. AND MOULÉ, Y.—(1957) *Exp. Cell Res.*, **13**, 181.
HOAGLAND, M. R., KELLER, E. E. AND ZAMECNIK, P. C.—(1956) *J. biol. Chem.*, **218**, 345.
KING, E. J.—(1932) *Biochem. J.*, **26**, 292.
KNOX, W. E.—(1951) *Brit. J. exp. Path.*, **32**, 462.—(1955) in S. P. Colowick and N. O. Kaplan, 'Methods in Enzymology'. New York (Academic Press), Vol. 2, p. 242.
LACASSAGNE, A.—(1933) *C. R. Soc. Biol., Paris*, **114**, 427.
LEE, N. D. AND WILLIAMS, R. H.—(1952) *Biochim. biophys. Acta*, **9**, 698.
MILLER, E. C.—(1951) *Cancer Res.*, **11**, 100.
Idem., AND MILLER, J. A.—(1947) *Ibid.*, **7**, 468.
MOORE, C. R., HUGHES, W. and GALLAGHER.—(1930) *Amer. J. Anat.*, **45**, 109.
MUTOLO, V. AND ABRIGNANI, F.—(1957) *Brit. J. Cancer*, **11**, 590.
NEEDHAM, J.—(1936) 'Terry Lectures'. London and New York (Cambridge University Press).
NOVIKOFF, A. B.—(1956) *Fed. Proc.*, **15**, 332.
PALADE, G. E.—(1955) *J. biophys. biochem. Cytol.*, **1**, 59.
PETERS, R. A.—(1936) *Proc. Roy. Soc.*, **121**, 587.
PORTER, K. R.—(1953) *J. exp. Med.*, **24**, 1424.
RUSKA, H., EDWARDS, G. A. AND CAESAR, R.—(1957) *Experientia*, **14**, 117.
SCHNEIDER, W. C.—(1945) *J. biol. Chem.*, **161**, 203.
WEBER, G. AND CANTERO, A.—(1955) *Cancer Res.*, **15**, 105.
Idem, ALLARD, CL., DE LAMIRANDE, G. AND CANTERO, A.—(1955) *Biochim. biophys. Acta*, **16**, 618.
WESTERFELD, W. W., RICKERT, D. A. AND HILFINGER, M. F.—(1950) *Cancer Res.*, **10**, 468.

THE *IN VITRO* AND *IN VIVO* ANALYSIS OF MAMMALIAN TUMOUR VIRUSES

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THE work of Ellerman and Bang (1908) and Rous (1911) on a leukaemia and sarcoma of chickens originally showed that viruses could be involved in the origin of neoplastic growth. Since then several tumour viruses have been isolated from birds and frogs, but in mammals, until relatively recently, the only viruses known to be associated with neoplasms were papilloma in rabbits and some other species, fibroma in rabbits, and mammary tumours in mice (Review in Oberling and Guerin, 1954). In 1951, Gross found that spontaneous leukaemia in AK mice could be transmitted by a virus. This observation has been amply confirmed by others (Woolley and Small, 1956; Furth *et al.*, 1956; Dulaney *et al.*, 1957; Hays and Beck, 1958), and in the last few years, viruses have been reported as responsible for some other types of leukaemia (Graffi, Bielka and Fey, 1956; Friend, 1957; Schoolman *et al.*, 1957), parotid tumours and sarcomas (Gross, 1953, 1955; Stewart 1955), and various other neoplasms in mice and hamsters (Stewart *et al.*, 1957, 1958; Eddy *et al.*, 1958*a*).

The above observations in mammals, except those of Stewart, Eddy *et al.*, were made by inoculating supernatants or filtrates from a tumour directly into a susceptible animal. The experiments were usually only successful if the inoculation was made into a host closely related genetically to the animal from which the extract was taken, and even under these conditions not all the tumours gave positive evidence for the presence of a virus. Even as regards the actual isolation of tumour viruses either from mice or from other mammals, this type of experiment has severe limitations. There may be a low virus content or virus inhibitors in some tumours. Such experiments by themselves can, of course, also give little direct information on cell-virus relationships and the mechanisms of tumour induction.

The basic problems relating to tumour viruses in mammals can be formulated as follows :—

(*1*) Are viruses involved in the origin of all tumours ? (*2*) How many types of viruses exist and to what extent are different types mutants of the same virus ? (*3*) How are the viruses transmitted, and what is the most effective means of preventing tumour induction ? (*4*) What is the nature of the cell-virus interaction and to what extent do tumour viruses resemble those of lysogenic systems ? (*5*) Are tumour viruses present in all animals ? Can they exist as proviruses that can be activated, or can they be formed *de novo*, by the chemical and physical agents known to induce the formation of tumours ? (*6*) What is the mechanism of the change produced by virus infection ? Is it direct or indirect, and is it sufficient

to produce the entire or only part of the neoplastic transformation ? Once the neoplastic state is produced, is the virus necessary for the maintenance of malignancy ? (*7*) How is the effectiveness of the immune response of the host related to the age difference in susceptibility to tumour induction by viruses ? (*8*) Can these viruses be used for genetic studies, and to establish systems of transduction and transformation ?

It seems clear that an answer to these questions, which may also be of clinical importance, can be given only by combining the *in vivo* and an *in vitro* analysis, and such a combined approach has been used in the present studies. It has been shown by Stewart *et al.* (1957, 1958) and Eddy *et al.* (1958*a*) that virus originating from a spontaneous lymphatic mouse leukaemia in strain AK can be maintained in tissue culture, and that when harvested from such cultures it can produce a variety of tumours in hosts not genetically closely related to the animal in which the tumour arose. We are indebted to Dr. Sarah Stewart and Dr. Bernice Eddy for sending us some of their line 3919, after it had been passaged twice in monkey kidney and 16 times in mouse embryo cultures. We have used this material, which they have termed polyoma virus, for *in vitro* and *in vivo* studies, and for comparison with virus obtained from other sources. We were at first concerned to establish methods for the *in vitro* characterization of tumour viruses (Sachs, Fogel and Winocour, 1959) and these methods have then been used in studies on tumour virus relationships.

EXPERIMENTAL

Plaques

We have routinely grown the polyoma virus on tissue culture monolayers of Swiss mouse embryo cells with a medium of 0·5 per cent lactalbumin hydrolysate in Earle's saline and 20 per cent horse serum. The cultures were grown in petri dishes in an incubator with a humidified atmosphere containing 10 per cent CO_2. The first culture of the material received from Stewart and Eddy showed a clear cytopathogenic effect (CPE) under our conditions of growth of the mouse embryo cells, and a CPE with this virus has also recently been reported by Eddy *et al.*, (1958*b*). As a result of the CPE the cells become granular, degenerate, and detach from the glass often in grape-like clusters. A CPE *in vitro* was also produced with this virus on two tissues from adults that were tested—mouse kidney and mouse bone marrow. By ultilising this effect on the cells in culture it has been possible to develop a plaque assay for this virus (Sachs, Fogel and Winocour, 1959) similar to that originally developed by Dulbecco and Vogt (1954) for viruses such as poliomyelitis. The generalised CPE was localised into foci under agar and the plaques appeared as small discrete foci of degenerate cells (Fig. 1).

The development of plaques makes possible a quantitative analysis of cell-virus relationships and the production of pure lines of virus.

Haemagglutination

We have also observed, as recently reported by Eddy *et al.* (1958*c*), that haemagglutination by the polyoma virus takes place at refrigerator (4°C) temperature. It does not haemagglutinate at room or higher temperatures. The virus has a specific spectrum of agglutinability with red blood cells (RBC) from different species (Sachs, Fogel and Winocour, 1959). Because of their availability guinea-

pig cells have been used for routine purposes. The pattern method was used and the tests read after 3 hours or overnight. 0·15 c.c. of a 1 per cent suspension of RBCs in saline was added to 0·5 c.c. of each dilution of virus, and the highest dilution giving a full agglutination of the RBCs (++++) was used as the end point. If the tubes are taken from the refrigerator after agglutination has occurred and placed at room temperature, the agglutination is destroyed. In experiments on elution and re-adsorption of the virus it was found that the virus elutes readily at 37°C, and that after elution the RBCs can again adsorb virus so that the receptors have not been destroyed. Temperature tests on the stability of the haemagglutinating particles showed that there is still full agglutination after 30 minutes at 56°C but almost none after 30 minutes at 66°C (Table I).

Haemagglutination inhibition was produced by antisera, and inhibition tests were carried out using 0·25 c.c. of antiserum, previously heated at 56°C for 30 minutes, and 0·25 c.c. of virus dilution containing 8 or 16 haemagglutinating units of virus. The virus-antiserum mixture was incubated for 30 minutes at 37° C.

In vitro *analysis of the growth of polyoma*

With the establishment of the plaque and haemagglutination techniques, it has become possible to make quantitative *in vitro* analyses on the growth of mammalian tumour viruses. Some experiments with the polyoma using these techniques have already been reported (Sachs, Fogel and Winocour, 1959), and these have shown: (*a*) that the virus multiplies in tissue culture, (*b*) that virus is released over a long period of time, (*c*) that a significant amount of virus produced remains cell associated and is not immediately released into the medium, and (*d*) that the data obtained by plaques follows that obtained by haemagglutination. The situation found in (*c*) is similar to that observed with viruses such as vaccinia (Overman and Tamm, 1957) and sheep pox (Plowright and Ferris 1958).

Additional data in support of these conclusions have been obtained with the polyoma. A further replicate of " total virus " in Experiment 1 (Sachs, Fogel and Winocour, 1959) has given haemagglutination titres of 1 : 8, 128, and 256 for days 1, 5 and 7 after infection. A plaque assay of " total virus " in Experiment 2, Series 2, has confirmed the haemagglutination data in showing that " free virus " was less than " total virus " throughout the experiment.

Virus of tumours from Gross

In order to study the characteristics of viruses that may be similar to the polyoma but that have never previously been in tissue culture, we have used virus from leukaemic mice obtained from Dr. Ludwick Gross. Dr. Gross kindly sent us some C3HGr mice that had developed leukaemia as a result of inoculation with his potent strain A virus (Gross, 1957). This strain, which produces primarily leukaemia in C3HGr mice, was developed by him by 14 successive inoculations into newborn C3HGr after its original isolation from a spontaneous lymphatic leukaemia in an AK mouse.

In order to study virus from this source *in vitro*, Swiss mouse embryo monolayers were inoculated with suspensions of (*1*) thymus, lymph nodes and spleen, and (*2*) brain, (from a leukaemic C3HGr mouse) using our standard culture medium. The suspensions were prepared by hand homogenisation after the organs

had been stored at — 20°C for 12 days. As a control, parallel cultures were inoculated with suspensions of thymus, lymph nodes and spleen, prepared in the same way, from two normal C3HGr mice that had also been sent to us by Dr. Gross.

In the first tissue culture passage, the medium was sampled at 3, 7 and 15 days after incubation, and already in this original passage the cultures inoculated with the leukaemic thymus, lymph nodes and spleen, gave a haemagglutination titre of 1 : 512 with guinea-pig RBCs, and a CPE on day 15. The 15-day fluid was used for a second passage and this gave a haemagglutination titre of 1 : 1024 on day 6 and a CPE on day 7. No haemagglutination or CPE was observed during these first two passages either in the cultures inoculated with brain from the leukaemic mouse or in those inoculated with organ suspensions from normal mice. Using haemagglutination, three tests showed a similarity between this virus and the polyoma. (*1*) It had a similar spectrum of agglutination with RBCs from different species, (*2*) it had a similar temperature stability at 56°C and 66°C (Table I), and (*3*) it was neutralised by antibodies against polyoma and *vice versa* (Table II).

These experiments therefore demonstrate (*a*) that there is a strong similarity between virus obtained from the two sources, (*b*) that a CPE can be obtained in the first passage in culture, and (*c*) that at least as regards this tumour, brain is not a better source of virus than the leukaemic organs. It should be noted that the original isolation of virus by Gross, and that of Stewart and Eddy which has now been in culture for at least 20 passages, were both made from spontaneous lymphatic leukaemia in AK mice.

Virus from chloroleukaemia

In addition to the material from lymphatic leukaemia, we have used extracts from a chloroleukaemia in mice. Dr. A. Graffi kindly sent us some mice (passage SOV 16) that had developed chloroleukaemia as a result of inoculation with a virus that had originally been isolated by him from the Ehrlich tumour (Graffi, Bielka and Fey, 1956). Using our standard medium, Swiss embryo monolayers were inoculated with suspensions of (*1*) lymph nodes and spleen, and (*2*) brain, from a leukaemic mouse with markedly green lymph nodes. The suspensions were made by hand homogenisation of organs that had been stored at — 20°C for 11 weeks. This material has now been through four passages in culture without any CPE. There was also no haemagglutination either by the medium or by extracts from cells of the culture. This virus, therefore, seems to behave differently in culture than that isolated from mice with spontaneous lymphatic leukaemia.

The induction of tumours by the polyoma virus and the production of virus by the tumour cells

The *in vivo* tumour inducing action of the polyoma was tested by inoculating newborn (less than 24 hours old) Swiss mice intraperitoneally or subcutaneously with 0·1 c.c. or 0·05 c.c. of virus taken from tissue culture, and we have observed, as also reported by Stewart *et al.* (1957, 1958) the induction of a great variety of tumours. Not all the tumours have yet been histologically analysed, but the most frequent so far encountered are kidney sarcomas, mucous gland tumours, mammary carcinomas, and hair follicle tumours. The Swiss mice used for these experiments,

TABLE I.—*Heat Inactivation of Haemagglutinating Particles*

Virus	Treatment	Virus dilution 1 : 2	4	8	16	32	64	128	256	512	1024	2048	Saline controls	
Polyoma	None	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	−	−	−
	56°C for 30 min.	+++	+++	+++	+++	+++	+++	+++	+++	+++	++	−	−	−
	66°C for 30 min.	−	−	−	−	−	+	+	−	−	−	−	−	−
Virus from Gross	None	+++	+++	+++	+++	+++	+++	+++	+++	+	−	−	−	−
	56°C for 30 min.	+++	+++	+++	+++	+++	+++	+++	+++	+++	−	−	−	−
	66°C for 30 min.	+++	+++	−	−	−	+	−	−	−	−	−	−	−

TABLE II.—*Cross Haemagglutination Inhibition with Sera from Mice Immunised with Polyoma and Virus from Gross*

Serum	Virus	Serum dilution 1 : 10	20	40	80	160	320	640	1,280	2,560	5,120	10,240	20,480	40,960	Virus controls	
Swiss antipolyoma	From Gross	−	−	−	−	−	−	−	−	−	−	−	−	+++	+++	+++
	Polyoma	−	−	−	−	−	−	−	−	−	−	−	−	+++	+++	+++
Swiss antivirus from Gross	From Gross	−	−	−	−	−	−	−	−	−	−	+	+++	+++	+++	+++
	Polyoma	−	−	−	−	−	−	−	−	−	−	−	+++	+++	+++	+++
Swiss normal	From Gross	−	−	−	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
	Polyoma	−	−	−	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++

and for the tissue cultures, are derived from an inbred line that has been brother-and-sister mated for at least 20 generations.

From our first passage of polyoma in culture, newborn Swiss were inoculated with tissue culture fluid harvested at 14, 21 and 28 days after infection of the monolayers. All three fluids produced tumours, despite the destruction of nearly all the cells of the monolayers by CPE at 28 days. A mouse, 13 weeks after inoculation with 14-day fluid is shown in Fig. 4, and a mouse, 11 weeks after inoculation with another fluid, in Fig. 2. Of the animals that survived more than one month after inoculation with various fluids, 61 out of 68 mice in a total of 17 litters have developed tumours in an observation period of 5 months. The earliest palpable tumours generally appeared at about 2 months after inoculation.

In addition to inoculation into newborn mice, one litter of four Swiss 7 days old, and one litter of four Swiss 14 days old were inoculated intraperitoneally with 0·1 c.c. of our first 14-day tissue culture fluid. Two of the four mice inoculated at 7 days of age have developed tumours in an observation period of 5 months, whereas no tumours have developed in this period in the 14-day old mice. The polyoma can therefore produce tumours in mice inoculated 7 days after birth, although the latency period for the earliest palpable tumours in these animals was 3½ instead of the usual 2 months. No tumour has developed in an observation period of 5 months in 50 Swiss mice inoculated as adults.

One litter of six newborn albino rats was inoculated with polyoma. Three survived more than one month and of these one developed sarcoma in both kidneys (Fig. 5) two months after inoculation.

There was a high early mortality in newborn mice inoculated with virus containing fluids, and those that survived frequently became dwarfed. In addition to dwarfing the affected animals showed a characteristic closure of the eyes. The difference between two litter mates, only one of which was inoculated with virus is shown in Fig. 3. The affected mice would either remain dwarfed and develop tumours, or recover from the drawfing and develop tumours later on.

Since the virus can induce tumours, it is obviously of interest to determine to what extent the cells of tumours induced with polyoma are able to produce virus. Extracts of tumours made by homogenisation with a motor driven homogeniser from five different mice did not give haemagglutination. Tumours were therefore trypsinised and the cells placed into culture using our standard medium. The cell inoculum was 10–12 $\times$ 10^6 cells per petri dish and the medium was tested by haemagglutination at various intervals after seeding. The results of four experiments from four different tumours are given in Table III, and they clearly show that tumour cells can produce virus under these conditions. It should be pointed out that with the medium used there was little or no growth of the tumour cells in culture, and the cells eventually degenerate.

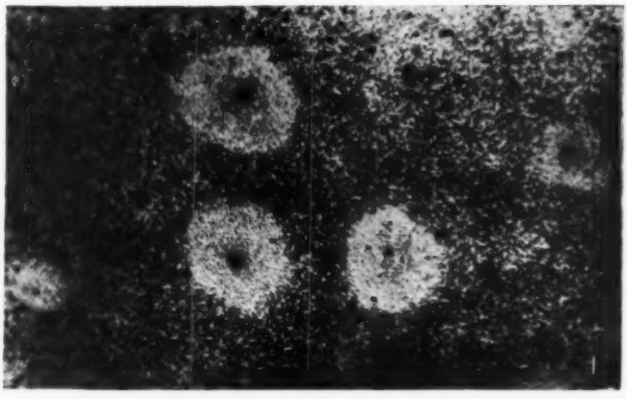
EXPLANATION OF PLATES

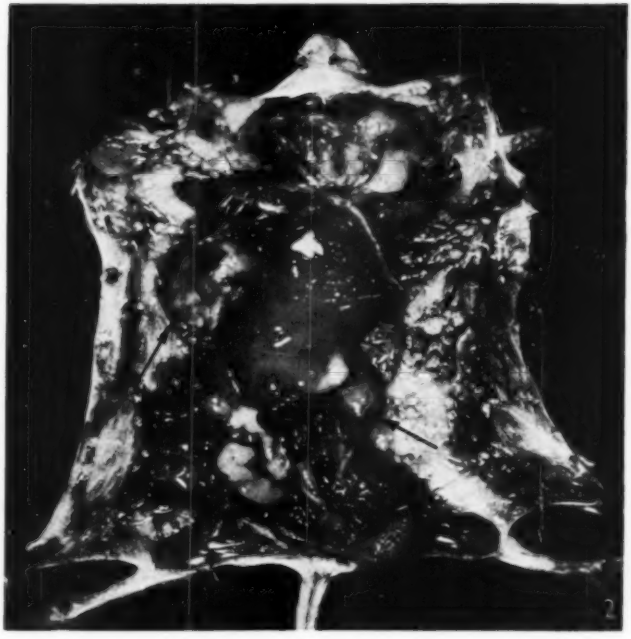
FIG. 1.—Plaques produced by polyoma virus on mouse embryo tissue culture. × 40.

Fig. 2 and 4.—Multiple tumours (some of which are shown by arrows) in Swiss mice inoculated with polyoma virus.

FIG. 3.—Two Swiss litter mates eight weeks old, showing a non-inoculated mouse and dwarfing of the litter mate inoculated with polyoma virus.

FIG. 5.—Rat with kidney tumours induced by polyoma virus.





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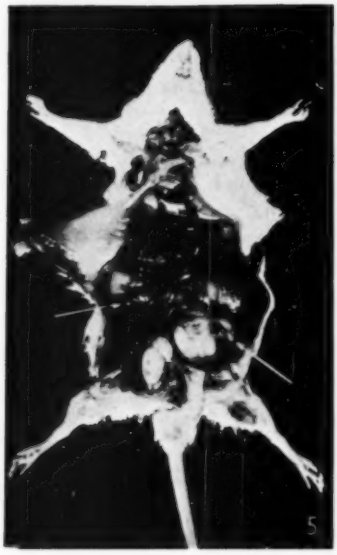
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TABLE III.—*Production of Virus by Tumour Cells in Culture*

Experiment No.	Days in culture	Haemagglutination titre 1 :
1	1	—*
	3	—
	7	128
2	8	128
	11	128
3	4	—
	8	1024
4	4	—
	8	2048
	10	2048

* — = Not higher than the control of normal time culture medium.

Cytopathogenic effect and tumour induction

The existence of (*1*) a CPE that produces cell death, and (*2*) tumour induction, raises the question to what extent these represent different reactions of cells to infection with the same virus. In order to answer this question it was first necessary to determine if both the CPE and the tumour are produced by the same virus. It has already been shown in the section on virus from Gross, that even the first passage in culture of virus from a tumour can produce a CPE. This has been further substantiated by tests made with two of the isolations shown in Table III. Swiss embryo monolayers were infected with the 7-day fluid from Experiment 1, and the 8-day fluid from Experiment 3, and both these isolations, made directly from tumour cells, gave a CPE in the first passage. No long period of adaptation is thus essential with this virus in order to produce a CPE on embryo cells in culture.

Neutralisation tests have given further evidence in favour of the supposition that both the CPE and the tumour are produced by the same virus. Antibodies in serum taken from tumour-bearing mice (see next section) must have been produced by the tumour-inducing virus, and it has been found that such antibodies can neutralise plaque formation by polyoma (Table IV). Such serum can also

TABLE IV.—*Inhibition of Plaque Formation by Serum from Tumour-bearing Mice*

			Average number of plaques per plate†
Experiment 1	Virus plus antiserum	1 : 10	53
	Virus plus normal serum	1 : 10	115
	Virus plus saline*		116
Experiment 2	Virus plus antiserum	1 : 10	0
		1 : 50	13
		1 : 250	18
		1 : 1250	42
	Virus plus normal serum	1 : 10	>200 ; confluent
		1 : 50	>200 ; confluent
		1 : 250	>200 ; confluent
		1 : 1250	>200 ; confluent
	Virus plus saline		>200 ; confluent

Neutralisation : Virus plus serum mixed 1 : 1 and held at 37°C for 1 hour in Experiment 1 and for 4 hours in Experiment 2.

* Earle's saline plus 0·5 per cent lactalbumin hydrolysate.

† Average of 4 replicate plates in Experiment 1 ; and of 3 replicate plates in Experiment 2.

neutralise the haemagglutinating particles. This demonstrates that virus that can produce CPE *in vitro* is neutralised by antibodies against virus that produces tumours *in vivo*. As has already been pointed out in the previous section, virus harvested after CPE produces tumours.

The high early mortality in young virus-injected mice, and the dwarfing effect in these mice after injection, furthermore suggest that a necrotic action of the virus may also occur *in vivo*. It thus seems that both an eventual death and a cell proliferating action can be produced by the same virus, and there is some evidence, which is now being studied quantitatively, that the polyoma can produce cell proliferation *in vitro* before CPE. It could be postulated that after virus infection a cell-virus relationship is established that changes the requirements of the infected cells and that if these new requirements are not met, e.g. as in the tissue culture medium used by us, the cells will degenerate, i.e. a CPE will be produced.

These results with the polyoma therefore provide an experimental analogy to the original observation of Duran-Reynals (1940) on the haemorrhagic disease produced by fowl tumour viruses. A CPE on cells in culture has also been reported for the virus of avian lymphomatosis (Sharpless, Defendi and Cox, 1958 ; Fontes *et al.*, 1958), and the possibility either of growth stimulation or degeneration as a result of infection with the same virus has been observed on the chorioallantoic membrane after infection with the Rous chicken virus (Prince, 1958) and with vaccinia (Overman and Tamm, 1957).

The production of antibodies in newborn, adult and tumour-bearing animals

Experiments on immunological tolerance have shown that the antibody-forming system in mice begins to function at about the time of birth (Billingham, Brent and Medawar, 1956), and it was therefore of interest to determine to what extent this is related to the age difference in susceptibility to tumour induction by virus. Using haemagglutination inhibition as a measure of the amount of antibody produced against the polyoma, it has been found that the response to virus infection differs in newborn and adult mice, and that this is reflected in a difference in the induction period before antibodies can be detected (Table V). In adults antibodies could be detected four days after inoculation whereas in newborn inoculated mice in this experiment, the titre was no higher than the control even at eight days. Tests made at later intervals after inoculation have shown that haemagglutination inhibition antibodies have always eventually been produced in mice inoculated as newborn (Table VI) and that there can be high titres even before any palpable tumours are observed. Particularly high titres have however been found in the animals with tumours

These data thus demonstrate that the high susceptibility of newborn mice to tumour induction, does not seem to be due to the production of a state of immunological tolerance to the virus.

It has been shown in a previous section that tumour cells are able to produce virus, and the high titres in the tumour-bearing mice are presumably due to this liberation of virus by cells of the growing tumour. Since the tumours continue to grow despite the presence of high titres of antibody, these data also indicate that antibodies produced against the virus are not effective in preventing the progressive growth of the tumour cells.

TABLE V.—*Induction Period in Newborn and Adult Swiss Mice Inoculated with Virus*

Days after inoculation	Age of mouse at inoculation	Haemagglutination-inhibition titre 1 :	Number of sera tested*
1	Adult	40	3 pooled
	Newborn	NT	
2	Adult	20	3 pooled
	Newborn	NT	
3	Adult	10	3 pooled
	Newborn	NT	
4	Adult	80	3 pooled
	Newborn	40	20 pooled
5	Adult	320	3 pooled
	Newborn	20	18 pooled
7	Adult	320	3 pooled
	Newborn	20	6 pooled
8	Adult	2560	3 pooled
	Newborn	40	8 pooled

All mice inoculated subcutaneously with 0·05 c.c. of polyoma virus with a haemagglutination titre 1 : 256.

NT = not tested.

* Different mice tested each day.

TABLE VI.—*Haemagglutination Inhibition Titres at Various Intervals after Inoculation of Virus into Newborn Swiss Mice*

Time (in weeks) after inoculation	Number of mice tested	Titre 1 :	Palpable tumours
2	2 pooled	320	−
2	7 pooled	1,280	−
3	2 pooled	640	−
3	2 pooled	2,560	−
4	2	2,560	−
4	1	10,240	−
5	2	5,120	−
13	2	>20,480	+
13	1	40,960	+
14	1	20,480	+
20	2	>5,120	+
22	1	>10,240	+
22	1	>20,480	+
22	1	40,960	+
22	1	>40,960	+

\+ = present.
− = absent.

Although the age difference in susceptibility thus does not seem to be due to immunological tolerance, it may still be due to the ability of the virus to multiply and re-infect other cells when inoculated into newborn, to a much greater extent than when inoculated into adults, and this point is being more fully investigated. A comparison of mice inoculated shortly after birth with virus, adult kidney cells followed by virus, and adult spleen cells followed by virus, has shown that at six weeks after virus infection, animals inoculated with the adult antibody-forming cells (spleen) and virus have a significantly greater percentage of survival and marked lack of dwarfing, in comparison to mice inoculated only with virus or with adult kidney cells (i.e. non-antibody-forming cells) and virus.

Antibodies in animals that develop spontaneous tumours

The finding of high antibody titres before palpable tumours can be observed, and even high titres in mice with obvious tumours, suggested the possibility that such a situation may also exist in animals which have not been purposely inoculated with virus, but which are known to develop spontaneous tumours. Haemagglutination inhibition tests with polyoma virus have therefore been made on mice of different ages belonging to the high leukaemic strain AKR, and the results are given in Table VII. None of 16 mice less than 23 weeks old had antibody levels higher than the control value, whereas 8 out of 12 mice more than 32 weeks old had considerable antibody titres against this virus. Tests on 20 Swiss mice, 34 to 56 weeks old and 20 C57BL mice 56 to 60 weeks old, have all shown no haemagglutination inhibition in excess of the control value found in younger animals.

TABLE VII.—*Haemagglutination Inhibition Tests with AKR Mice*

Age in weeks	Number of tests*	Titre 1 :
13	6	20
15	1	10
15	5	20
17	2	20
23	2	20
32	1	320
32	1	640
32	4	1280
34	1	20
34	1	1280
35	2	20
36	1	10
36	1	2560

* Each test represents the result from a different mouse.

These data show that AKR mice can carry the polyoma, and furthermore that the detection of antibodies may be a useful guide (*a*) to the existence of tumour virus in animals, and (*b*) to the eventual development of tumours. The absence of high titres in four of the other older AKR suggests either that these animals will not develop tumours, or that although a high antibody titre may frequently be correlated with eventual tumour development, this correlation may perhaps not exist in every animal. The existence of antibodies is therefore being further studied in relation to histological examination of the tissues for malignancy in AKR, and in animals that develop other types of tumours.

In addition to the experiments on AKR, haemagglutination inhibition tests were made with serum from seven C3H mice with large and five with small spontaneous mammary tumours. Of these four with large tumours showed antibody titres of 1 : 80, 320, 1280, and 2560 respectively. Some C3H mice may therefore also carry the polyoma, and since the C3H used was known to carry the mammary tumour virus (milk agent), there may perhaps be some relationship between the mammary virus and polyoma.

The transmission of polyoma virus

One of the basic questions relating to tumour viruses is how the viruses are transmitted. In order to obtain information on this point, experiments were

carried out with the polyoma on the possibility of virus transmission. (*1*) between litter mates, (*2*) from virus injected litters to their mother, and (*3*) from a virus injected mother to her litter. Haemagglutination inhibition was used as a test for the presence of virus. The results are given in Table VIII, and the data clearly show that virus can be transmitted under all three conditions. It should be noted that in all these tests both the injected and non-injected animals were kept in the same cage. In the experiment in which non-injected animals were kept in the same room but in a different cage (Table VIII) there has so far been no evidence of virus transmission. We have also so far found no haemagglutination inhibition antibodies in the sera of the people who have been in contact with the polyoma in the tissue culture, haemagglutination, and animal experiments.

TABLE VIII.—*Virus Transmission in Swiss Mice*

Serum from :	Number of mice	Source of infection	Tested (days) after contact	Haemagglutination inhibition titre 1 :
Non-inoculated litter mates	3 pooled	Newborn litter mates inoculated i.p.*	45	1280
Non-inoculated litter	5 pooled	Mother inoculated i.p. 16th day of pregnancy	27	320
Mother	1	Litter inoculated s.c.	15	20
,,	1	Litter inoculated s.c. and i.p.	15	20
,,	1	Litter inoculated s.c. and i.p.	15	20
,,	1	Litter inoculated s.c.	21	80
,,	1	Litter inoculated i.p.	37	80
,,	1	Litter inoculated i.p.	50	320
,,	1	Litter inoculated s.c.	50	640
,,	1	Litter inoculated i.p.	50	640
,,	1	Litter inoculated i.c.	50	640
Adult	3 pooled	Inoculated with normal tissue culture medium as newborn and kept in same room as virus-inoculated mice	59	40
Control	5	None	—	40

* i.p. = Intraperitoneal, s.c. = subcutaneous, and i.c. = intracranial.

It can thus be seen that a tumour virus can be transmitted under certain conditions from animal to animal, and the exact route of infection, i.e. whether from urine, faeces, saliva, milk, transplacental, etc., can now be tested.*

The isolation of virus from human tumours

The results described above suggested that by using a similar *in vitro* and *in vivo* approach it may be possible to determine the role of viruses in the origin of human tumours. The methods developed for the mouse are therefore being applied to material from patients. In order to avoid contamination, the experiments are being carried out by one of us in a separate tissue culture cubicle, at a time when he is not handling virus isolated from mice. Human material has been inoculated onto monolayers of Swiss mouse embryos using our standard medium. Cultures consisting of bone marrow from two patients (one with

* *Note added in proof.* Isolations from five animals have shown that the polyoma can be excreted in the faeces, urine and saliva.

myeloid and one with stem cell leukaemia) on Swiss embryo cells have given an effect on the cultures, and the *in vivo* tumour-inducing activity of this material is now under examination.

DISCUSSION

It can be seen from the above results, that by combining an *in vitro* with an *in vivo* approach, experimental systems can be established in order to answer some of the basic problems relating to the role of viruses in the origin of mammalian tumours. In addition, the establishment of these systems is also of value for the actual isolation and characterisation of tumour viruses.

The development of the plaque and haemagglutination techniques has made it possible to begin a quantitative analysis of cell-virus relationships, which is a basic pre-requisite for an understanding, at the cellular level, of the role of viruses in tumour formation. For this study, the growth of polyoma on mouse embryo monolayers has been used as a model. The finding in this system that a cytopathogenic effect *in vitro* and tumour induction *in vivo* are produced by the same virus, shows that infection with tumour virus can produce under different conditions either growth stimulation or eventual cell degeneration.

The use of *in vitro* tests such as haemagglutination inhibition, has shown that these tests can readily provide information on the presence of tumour virus in an animal, the development of tumours induced by virus, and the transmission of virus from one animal to another.

One of the questions raised in the present studies is the relationship between leukaemia, polyoma, and mammary tumour virus (milk agent), and whether a single or a group of viruses is involved in the origin of multiple tumours. It has been shown that there is a strong similarity between the virus obtained from Gross and polyoma. In addition, the findings (*1*) that high antibody titres against polyoma can exist in some C3H mice (known to carry the mammary tumour virus) with spontaneous mammary tumours, and (*2*) that a high frequency of mammary carcinomas is induced in Swiss mice inoculated with the polyoma, suggest that some C3H mice may carry the polyoma and that this virus may perhaps be related to the mammary tumour virus. The observation of Gross (1955) that extracts from normal C3H tissues can induce parotid tumours and subcutaneous sarcomas when inoculated into newborn C3H mice, also suggests that C3H may carry the polyoma. The fact that the mammary tumour virus cultured on plasma clots with chick fibroblasts (Pikovski, 1953) produced under these conditions only mammary tumours in C57BL mice and C57BL and RIII hybrids, does not necessarily rule out a relationship between this virus and the polyoma. The conditions of the culture system, and possibly even the test animals, may not have been the best to demonstrate the full potentialities of the virus after passage in culture. Mammary tumour virus is therefore being cultured using the same conditions as for the polyoma.

Since with the development of the plaque technique it is now possible to isolate pure lines of virus from different tumours, such isolations are being tested for the types of tumour that they produce in animals. The use of such pure lines in *in vivo* infection experiments should be able to demonstrate conclusively to what extent mammary tumours, leukaemia, and other types of tumours are produced by the same mutants, or quite different strains of virus. The establishment of such pure lines could also provide material for a genetic analysis of these animal

viruses. The plaque isolates will thus be able to show to what extent the situation in mice resembles that in chickens where various types of tumours seem to be produced by a group of related viruses (Beard, 1957).

It has been shown that tumour cells can produce virus and this has been demonstrated by placing the cells in culture. This technique may have the additional advantage of providing the most satisfactory method of isolating virus from different tumours and it is now being applied to the studies on humans. In addition, these results suggest that there exists in these tumours some stable form of cell-virus relationship. Evidence for such a relationship has also been obtained for the Rous chicken virus (Rubin, 1955), and it also seems to exist in avian myeloblastosis (Beaudreau *et al.*, 1958). This raises the further possibility that mammalian tumour viruses may prove to be of value for the study of transduction of genetic characters, e.g. antigens, in mammalian cells where there already exist some good genetic markers. There may also exist some further analogues to the fibroma-myxoma transformation (Kilham, 1958). The study of mammalian tumour viruses may therefore be of value not only for an understanding of the origin of tumours, but also for an understanding of some other basic properties of viruses and cells.

SUMMARY

1. A combined *in vitro* and *in vivo* analysis has been made of virus (virus from Gross and polyoma virus) originally isolated from mice with spontaneous lymphatic leukaemia. An *in vitro* study has also been made of a virus from a chloroleukaemia in mice. The *in vitro* analysis has been based on the use of tissue culture monolayers and the development of a plaque and haemagglutination technique. These techniques make possible a quantitative *in vitro* analysis of tumour virus relationships, and the development of plaques further makes possible the production of pure lines of virus. *In vitro* methods can also be applied to the study of human tumours.

2. Using the growth of polyoma on mouse embryo monolayers as a model system, it has been shown that the virus multiplies in tissue culture, that virus is released over a long period of time, that a significant amount of virus produced remains cell associated, and that the data obtained by plaques follows that obtained by haemagglutination.

3. Studies on virus from Gross have demonstrated that there is a strong similarity between a virus from this source and the polyoma, and that at least as regards this tumour, brain is not a better source of virus than leukaemic organs.

4. Virus from chloroleukaemia seems to behave differently in culture than that isolated from mice with spontaneous lymphatic leukaemia.

5. The polyoma when inoculated into newborn Swiss mice produced a range of tumours, the most frequent so far being kidney sarcomas, mucous gland tumours, mammary carcinomas, and hair follicle tumours. The earliest palpable tumours usually appeared about 2 months after inoculation, and in an observation period of 5 months, tumours developed in 90 per cent (61/68) of the inoculated mice. Tumours with a longer latent period were produced in two Swiss mice inoculated at seven days of age, but none in mice inoculated at fourteen days or later. Kidney sarcomas were found two months after inoculation of virus into a newborn rat.

6. Studies on tumour cells in culture have shown that malignant cells can produce virus.

7. It has been shown that a cytopathogenic effect on cells *in vitro*, and tumour induction *in vivo*, are produced by the same virus.

8. Studies on the immune response of the host have shown that the high susceptibility of newborn animals does not seem to be due to the induction of a state of immunological tolerance to the virus. All animals inoculated as newborn eventually develop haemagglutination inhibition antibodies (even before observable tumours) and such antibody titres are particularly high in animals with palpable tumours.

9. AKR mice of different ages (which were not inoculated with virus) were tested for haemagglutination inhibition antibodies against polyoma. None of 16 mice less than 23 weeks old had significant levels of antibody, whereas 8 out of 12 mice more than 32 weeks old had considerable antibody titres. This has shown that AKR mice carry the polyoma, and that the detection of antibodies may be a useful guide to the existence of tumour viruses, and possibly also to the eventual development of tumours.

10. No significant haemagglutination inhibition titres were found in 20 normal Swiss mice 34 to 56 weeks old or in 20 C57BL mice 56 to 60 weeks old.

11. Studies on the transmission of the polyoma have shown that the virus can be transmitted from inoculated to non-inoculated litter mates, from an inoculated mother to her non-inoculated litter, and from an inoculated litter to their non-inoculated mother.

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REFERENCES

BEARD, J. W.—(1957) *Ann. N.Y. Acad. Sci.*, **68**, 473.

BEAUDREAU, G. S., BECKER, C., SHARP, D. G., PAINTER, J. C. AND BEARD, J. W.—(1958) *J. nat Cancer Inst.*, **20**, 351.

BILLINGHAM, R. E., BRENT, L. AND MEDAWAR, P. B.—(1956) *Phil. Trans.*, s.B. **239**, 357.

DULANEY, A. D., MAXEY, M., SCHILLIG, M. G. AND GOSS, M. F.—(1957) *Cancer Res.*, 17, 809.

DULBECCO, R. AND VOGT, M.—(1954) *J. exp. Med.*, **99**, 167.

DURAN-REYNALS, F.—(1940) *Yale J. Biol. Med.*, **13**, 77.

EDDY, B. E., STEWART, S. E., YOUNG, R. AND MIDER, G. B.—(1958*a*) *J. nat. Cancer Inst.*, **20**, 747.

Iidem AND BERKELEY, W.—(1958*b*) *Proc. Soc. exp. Biol. N.Y.*, **98**, 848.

Idem, ROWE, W. P., HARTLEY, J. W., STEWART, S. E. AND HUEBNER, R. J.—(1958*c*) *Virology*, **6**, 290.

ELLERMAN, V. AND BANG, O.—(1908) *Zbl. Bakt.*, **46**, 595.

FONTES, A. K., BURMESTER, B. R., WALTER, W. G. AND ISELER, P. E.—(1958) *Proc. Soc. exp. Biol. N.Y.*, **97**, 854.

FRIEND, C.—(1957) *J. exp. Med.*, **105**, 307.

FURTH, J., BUFFETT, R. A., BANASIEWICZ-RODRIGUEZ, M. AND UPTON, A. C.—(1956) *Proc. Soc. exp. Biol. N.Y.*, **93**, 165.

GRAFFI, A., BIELKA, H. AND FEY, F.—(1956) *Acta Haemat.*, **15**, 145.

GROSS, L.—(1951) *Proc. Soc. exp. Biol. N.Y.*, **76**, 27.—(1953) *Ibid.*, **83**, 414.—(1955) *Ibid.*, **88**, 362.—(1957) *Ibid.*, **94**, 767.

HAYS, E. F. AND BECK, W. S.—(1958) *Cancer Res.*, **18**, 676.

KILHAM, L.—(1958) *J. nat. Cancer Inst.*, **20**, 729.

OBERLING, C. AND GUERIN, M.—(1954) *Advanc. Cancer Res.*, **2**, 353.

OVERMAN, J. R. AND TAMM, I.—(1957) *Virology*, **3**, 173.

PIKOVSKI, M. A.—(1953) *J. nat. Cancer Inst.*, **13**, 1275.
PLOWRIGHT, W. AND FERRIS, R. D.—(1958) *Brit. J. exp. Path.*, **39**, 424.
PRINCE, A. M.—(1958) *Virology*, **5**, 435.
ROUS, P.—(1911) *J. exp. Med.*, **13**, 397.
RUBIN, H.—(1955) *Virology*, **1**, 445.
SACHS, L., FOGEL, M. AND WINOCOUR, E.—(1959) *Nature, Lond.*, in press.
SHARPLESS, G. R., DEFENDI, V. AND COX, H. R.—(1958) *Proc. Soc. exp. Biol. N.Y.*, **97**, 755.
SCHOOLMAN, H. M., SPURRIER, W., SCHWARTZ, S. O. AND SZANTO, P. B.—(1957) *Blood*, **12**, 694.
STEWART, S. E.—(1955) *J. nat. Cancer Inst.*, **15**, 1391.
Idem, EDDY, B. E., GOCHENOUR, A. M., BORGESE, N. G. AND GRUBBS, G. E.—(1957) *Virology*, **3**, 380.
Iidem AND BORGESE, N. G.—(1958) *J. nat. Cancer Inst.*, **20**, 1223.
WOOLLEY, G. W. AND SMALL, M. C.—(1956) *Cancer*, **9**, 1102.

THE *IN VITRO* AND *IN VIVO* ANALYSIS OF MAMMALIAN TUMOUR VIRUSES

The Haemagglutinating System of the Polyoma Virus

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Since the classical work of Ellerman and Bang (1908) on chicken leukaemia and of Rous (1911) on chicken sarcoma first demonstrated the viral origin of certain tumours, such studies have been extended to various species including mammals (Rous, 1935 ; Oberling and Guerin, 1954 ; Gross, 1958). It was shown in our previous investigations (Sachs, Fogel and Winocour, 1959a ; Sachs *et al.*, 1959b, Winocour and Sachs, 1959), that basic questions relating to the role of viruses in the origin of mammalian tumours can best be answered by combining an *in vivo* with an *in vitro* analysis, and that by using such a combined approach it is possible to establish particularly favourable experimental systems. One of the viruses used in these studies was the polyoma virus (Eddy *et al.*, 1958a) originally isolated from AK mice with lymphatic leukaemia (Gross, 1958 ; Stewart, Eddy and Borgese, 1958). This virus can agglutinate red blood cells from various species at refrigerator (4° C.) temperature (Eddy *et al.*, 1958*a* ; Sachs, Fogel and Winocour, 1959a), and it was shown that this ability for haemagglutination can be used both for an *in vitro* virus identification, and to determine various aspects of virus behaviour (Sachs, Fogel and Winocour, 1959a ; Sachs *et al.*, 1959b).

In order to establish more fully the characteristics of this virus, and to compare these with non-tumour forming viruses, a further investigation was carried out on its haemagglutinating system. The present studies are therefore concerned with the properties of the haemagglutinating particles, the formation of haemagglutination inhibition antibodies after virus inoculation in relation to tumour formation, and the existence of such antibodies in AKR mice.

MATERIALS AND METHODS

Animals

The Swiss mice used for the tissue cultures and virus inoculations into mice, are derived from a line that has been brother × sister mated for at least 20 generations. The AKR mice are derived from breeding pairs originally obtained from the Roscoe B. Jackson Memorial Laboratory, Bar Harbor, and the golden hamsters from a randomly bred colony originating in this country.

Virus

The polyoma virus stocks used for the present experiments were from lines IL 1–6 ; and from a line isolated (Sachs *et al.*, 1959b) from leukaemic C3HGr

mice kindly supplied by Dr. Ludwick Gross and inoculated by him with his strain A leukaemic virus (Gross, 1957). The IL lines, which were obtained after culturing of tumour cells (Sachs *et al.*, 1959b), consist of isolations 1 and 4 from mouse parotid tumours, 2 and 5 from mouse mammary adenomas, 3 from a mouse mammary adenocarcinoma, and 6 from a rat kidney sarcoma. The tumours used for these isolations were induced with virus derived from strain 3919 (Steward, Eddy and Borgese, 1958) kindly supplied by Dr. Sarah E. Stewart and Dr. Bernice E. Eddy. All virus stocks were made and used in a medium consisting of 0·5 per cent lactalbumin hydrolysate in Earle's saline and 20 per cent horse serum.

Haemagglutination

Haemagglutination tests were routinely made with guinea-pig red blood cells, using 0·15 c.c. of a 1 per cent suspension in saline added to 0·5 c.c. of each dilution of virus. The saline was adjusted with NaOH to pH 6·8–7·2. Virus dilutions were made in saline. Blood was taken from the heart in Alsever's solution, and used on the same day. Sedimentation of the controls was found to be inadequate, if the red blood cells were kept more than 4 days after collection. In order to standardise the 1 per cent suspension, 1 c.c. of the cell suspension was mixed with 4 c.c. of double distilled water and after complete haemolysis the concentration of haemoglobin measured in a Coleman Junior spectrophotometer at a wavelength of 545 mμ. An optical density of 0·26 was taken to be equivalent to a 1 per cent suspension of red blood cells. The pattern method of agglutination (Salk, 1944) was used, and the tests read after 3 hours or overnight at 4° C. Since the agglutination is destroyed at room temperature, the tests were read immediately after the tubes were taken from the refrigerator.

Haemagglutination inhibition

Inhibition tests were made using 0·25 c.c. of antiserum, previously heated at 56° C. for 30 minutes, and 0·25 c.c. of virus suspension containing 16 haemagglutinating units of virus. Complete inhibition was used as the end point. Before adding the red blood cells, the virus antiserum mixture was incubated for 30 minutes at 37° C. Serum from normal Swiss mice gave inhibition titres up to 1 : 40.

EXPERIMENTAL

The haemagglutination spectrum

The polyoma has a specific haemagglutination spectrum when tested with red blood cells from various species (Sachs, Fogel and Winocour, 1959a). The results with two lines of this virus, and a comparison with the spectrum of some other haemagglutinating viruses (data from Smadel (1948) and Burnet and Stone (1946)) are shown in Table I. The two lines of polyoma used were line IL–3, and the line isolated from a C3HGr leukaemic mouse (Sachs *et al.*, 1959b). Although we have so far found no haemagglutination with cells from our hamsters, this species has been reported as positive by Eddy *et al.* (1958a). This may be due to variation within the species or the existence of virus mutants. The most rapid reading was given by cells from the frog *Hyla arborea*, where there was complete sedimentation after 30 minutes at 4° C. in contrast to the usual 3 hours with cells from other species. The cells from this frog also gave the highest titre (Sachs, Fogel and

TABLE I.—*Haemagglutination Spectrum of Polyoma Compared to some other Haemagglutinating Viruses*

Red blood cells	Polyoma	Virus from Gross	Influenza A	Influenza B	Newcastle disease	Vaccinia	Pneumonia virus of mice	Ectromelia
Guinea-pig	+	+	+	+	+	+	−	−
Rat	−	−	±	±	+	±	−	−
Mouse	+	+	±	±	+	±	+	+
Hamster	−*	−	N.D.	N.D.	N.D.	N.D.	+	N.D.
Dog	+	+	+	N.D.	+	N.D.	−	N.D.
Cat	−	−	−	+	−	+	−	N.D.
Human	+	+	+	+	+	±	−	−
Monkey	+	+	−	N.D.	−	−	N.D.	N.D.
Horse	−	−	−	±	−	−	N.D.	N.D.
Cow	−	−	−	+	+	−	N.D.	−
Sheep	+	+	±	±	±	−	−	−
Goat	−	−	±	+	+	−	N.D.	N.D.
Chicken	+	+	+	+	+	+	−	+
Turkey	+	+	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
Goose	−	−	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
Pigeon	−	−	N.D.	N.D.	N.D.	+	N.D.	+
Duck *Anas moscant*	+	+	+	+	+	+	N.D.	−
Duck *Anas boschas*	−	−						
Frog *Hyla arborea*	+	+	+	+	+	−	N.D.	N.D.
Frog *Bufo viridis*	−	−						

+ = positive. − = negative. N.D. = No data. * = reported as + by Eddy *et al.* (1958*a*).

Winocour, 1959a), and if available in quantity would be the most suitable for haemagglutination. Because of availability, guinea-pig red blood cells have been used in all the following experiments.

The effect of pH

In order to test the effect of pH, haemagglutination was carried out in 0·15 M phosphate buffer at different pH from 5·4–8·4. At the higher (7·7–8·4) and lower pH (5·4 and 6·0), there was not a complete sedimentation of cells in the buffer control. It is clear (Table II), that the virus can agglutinate over a wide range of pH, although there is a lower titre at the lower pH. These tests and all the following experiments on the properties of the haemagglutinins were made with a virus stock of line IL–3, obtained by harvesting the fluid from cultures of tumour cells (Sachs *et al.*, 1959b).

Adsorption and elution from red blood cells

Adsorption and elution experiments were carried out to determine the possession by the virus of receptor destroying enzyme, and the speed of the reactions. A final concentration of 2 per cent red blood cells was used with the stock virus fluid diluted 1 : 8. Adsorption was carried out at 4° C. and elution at 37° C. For the experiments shown in Table III, after adsorption of the virus, the cells were sedimented by centrifugation in a clinical centrifuge at 1500 r.p.m. at 4° C., and then washed twice by resuspending in the same amount of saline and centrifugation at 4° C. Each elution was for 1 hour at 37° C, followed by centrifugation at 37° C. to sediment the red blood cells. The cells were then resuspended in the same amount of saline for the next elution. After 4 such elutions the cells were tested for their ability to again adsorb virus.

TABLE II.—*Effect of Differences in pH on Haemagglutination*
Virus dilution

TABLE II.—*Effect of Differences in pH on Haemagglutination*

pH	Virus dilution 1 : 2	4	8	16	32	64	128	256	512	1024	2048	4096	8192	Control
5·4	+++	+++	+++	+++	+++	+++	+++	+++	±	±	±	±	±	±
6·0	+++	+++	+++	+++	+++	+++	+++	+++	+++	±	±	±	±	±
6·4	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	–	–	–	–
6·8	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	++	–	–	–
7·2	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	++	+	–	–
7·7	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	++	±	±
8·0	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	±	±
8·4	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	++	++	±	±

TABLE III.—*Virus Adsorption and Elution from Red Blood Cells*

	Virus dilution 1 : 8	16	32	64	128	256	512	1024	2048	4096	8192	Saline control
Initial (virus containing) t.c.f.	+++	+++	+++	+++	+++	+++	+++	+++	+++	++	–	–
After adsorption with r.b.c.	–	–	–	–	–	–	–	–	–	–	–	–
Saline after 1st washing of r.b.c.	–	–	–	+	+	–	–	–	–	–	–	–
Saline after 2nd washing of r.b.c.	–	–	–	–	–	–	–	–	–	–	–	–
1st eluate	+++	+++	+++	+++	+++	+++	+++	+++	+	–	–	–
2nd eluate	+++	+++	+++	+++	+++	+++	+	–	–	–	–	–
3rd eluate	+++	+++	+++	++	++	++	+	+	–	–	–	–
4th eluate	+++	++	++	+	+	+	–	–	–	–	–	–
Initial t.c.f. with r.b.c. used for adsorption and elution	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	++	+

t.c.f. = tissue culture fluid. r.b.c. = red blood cells.

TABLE IV.—*Haemagglutination Titres After Centrifugation at* 105,000 *g*

Time of centrifugation	Virus dilution 1 : 2	4	8	16	32	64	128	256	512	1024	2048	4096	8192	Saline control
None (control)	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+	–	–	–
Supernatant after 1 hour	+++	+++	+++	+++	+++	–	–	–	–	–	–	–	–	–
Sediment after 1 hour	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	–	–	–
Supernatant after 3 hours (top 2 c.c.)	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Supernatant after 3 hours (bottom 10 c.c.)	+++	+++	+++	–	–	–	–	–	–	–	–	–	–	–
Sediment after 3 hours	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	–	–	–

The results given in Table III show that saline washing does not remove the haemagglutinins from the red blood cells, and that even after 4 elutions at 37° C. there are still some agglutinins attached to the cells. They further show that the cells used for elution can again adsorb virus, so that polyoma does not seem to have a receptor destroying enzyme such as is found in influenza, mumps and NDV (Hirst, 1952).

To determine the speed of the reactions, the suspension was sampled at various intervals after the beginning of adsorption and elution. Each test was made in a different tube, the suspension centrifuged at the appropriate times, and the

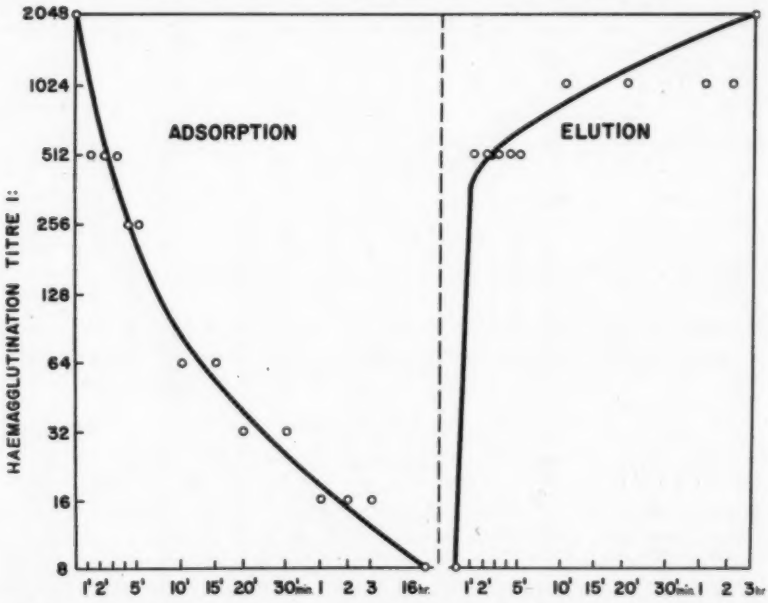

FIG. 1.—Adsorption and elution curves for polyoma haemagglutinins on guinea-pig red blood cells.

haemagglutination titre assayed. The results (Fig. 1) show a rapid adsorption and a rapid elution. The titre dropped from the original 2048 to 64 after an adsorption time of 10 minutes, and during elution, the titre rose to 512 after 1 minute and to 1024 after 10 minutes. A rapid adsorption was also found by Hirst (1942) for influenza virus, although elution with influenza was not as rapid as with polyoma. The polyoma did not show spontaneous elution at 4° C.

Sedimentation at 105,000 *g*.

It was found that supernatant fluid after centrifugation for 1 hour at 105,000 *g* produced tumours when inoculated into newborn Swiss mice. Experiments were therefore carried out on the sedimentation of the virus at this speed. Stock virus was first centrifuged at 9000 *g* for 10 minutes in a Servall Angle Centrifuge, and the supernatant then centrifuged in a Spinco Model L Preparative Ultracentrifuge

with a No. 40 Rotor at 40,000 r.p.m. average = 105,000 *g*. The supernatant and sediment were sampled for haemagglutination titre from 12 c.c. of virus stock after 1 hour, and from another 12 c.c. after 3 hours centrifugation in the Spinco. The sediment was resuspended in 5 c.c. of medium without serum.

The results, Table IV, show that after 1 hour haemagglutinins have sedimented. Samples of 1 c.c. from supernatant after 1 hour, and from the top 2 c.c., the bottom 10 c.c. and the resuspended sediment after 3 hours centrifugation, were inoculated intraperitoneally into adult Swiss mice. Haemagglutination inhibition antibodies in the pooled serum from 2 mice per group were determined 14 days after inoculation and these gave titres of 1 : 1280, 1280, 1280 and 2560 respectively. The supernatants thus still contain some virus particles. The size of the particles and possible existence of aggregates is being checked by electron microscope observations. Filtration of stock virus through a Selas 02 candle filter did not decrease the haemagglutinin titre. The production of tumours by the supernatant after 35 minutes at 105,000 *g* has also been reported by Buffett *et al.* (1958).

Ultraviolet inactivation

The inactivation of polyoma by ultraviolet was examined in order to determine the degree of inactivation of the haemagglutinating particles and to compare this with effects on infectivity. Infectivity was measured on tissue cultures and in animals. Stock virus was centrifuged in a Servall Angle Centrifuge at 9000 *g* for 10 minutes, and for each test, 1 c.c. of the supernatant was irradiated in a watch glass with a 15 watt Philips TUV germicidal lamp placed at a distance of 24 cm. from the virus containing fluid. This gives a radiation intensity of 30 ergs/mm.^{2}/second. The watch glasses were agitated every 10 minutes, and before titration any medium loss by evaporation was added to make the original 1 c.c. The haemagglutination titres obtained after various exposures (Table V) show that there was a decrease of one dilution after 60 minutes, a further decrease by one dilution after 100 minutes, and that there was still some agglutination after 150 minutes.

To compare the effect on haemagglutination with effects on infectivity, virus after various periods of irradiation was inoculated onto mouse embryo monolayers. The monolayers were grown in petri dishes in a medium of 0·5 per cent lactalbumin hydrolysate in Earle's saline and 20 per cent horse serum, in an incubator with a humidified atmosphere containing 10 per cent CO_2. Each test was made on a pool from two petri dishes, and each petri dish containing 4 c.c. of medium. 0·3 c.c. of virus was adsorbed for 3 hours, without washing, and the medium changed at 2, 5, 8 and 11 days later. The haemagglutination titres per 0·5 c.c. were determined on days, 5, 8 and 11, and the cultures were also examined for a clear cytopathogenic effect. It can be seen (Table VI) that virus irradiated for 30 minutes before inoculation on to monolayers gave after 11 days growth a haemagglutination titre of 256 (in contrast to a titre of 2048 for non-irradiated virus) and that after 60 minutes irradiation the titre was only 32 in comparison to 16 for normal tissue culture fluid. After an exposure of 90 minutes or longer there were no haemagglutination titres higher than those of the normal tissue culture fluid. Since the yield of haemagglutinins in tissue cultures is correlated with the amount of infective virus in the inoculum, these results show that, as in the case of influenza

(Henle and Henle, 1947), infectivity of the polyoma virus is more sensitive to ultraviolet than haemagglutination. There was also a correlation between haemagglutinin yield and the cytopathogenic effect on the culture (Table VI).

The infectivity of the irradiated virus was further measured by determining the haemagglutination inhibition antibody titre at 2 and at 4 weeks after inoculation of 0·05 c.c. subcutaneously into 2-day-old Swiss mice. Each test was made with the pooled serum from 2 or 3 mice, and the results are shown in Table VI. The increase in titre between 2 and 4 weeks indicates that there may still have been some infective virus after 150 minutes' irradiation.

Temperature inactivation

In order to determine stability at different temperatures, experiments were carried out on the temperature inactivation of the haemagglutinins and infectivity. The tests for infectivity and cytopathogenic effect were made in the same way as for the ultraviolet experiments, with samples from the same virus stock. There was no decrease in haemagglutination titre (Table VII) after 30 minutes at 56° C., a decrease at higher temperatures, and no significant titre with the method used after 30 minutes at 66° C. or 15 minutes at 70° C. The infectivity tests on the cultures (Table VIII) showed no decrease in yield of haemagglutinins after 30 minutes at 56° C., a decrease at higher temperatures, and that there was still some infectious virus after 30 minutes at 66° C. and 15 minutes at 70° C. The existence of some haemagglutination titres lower than the control of 32 in column 2 of Table VIII can be explained by the inactivation at these temperatures of the agglutinating antibody in the horse serum of the medium.

The haemagglutination inhibition titres after inoculation into mice (Table VIII) also show the high temperature stability of the virus under the present conditions, and that there may still have been some infective virus even after 30 minutes at 70° C. The existence of some infective virus after 30 minutes at 70° C. has also been reported by Eddy, Stewart and Grubbs (1958b). In tests on the stability of the haemagglutinin at 37° C., it was found that there was no loss of titre after 8 weeks at this temperature.

It can be seen from the above that whereas in the ultraviolet inactivation tests infectivity was clearly more sensitive than haemagglutination and this has also been found with inactivation by formalin (Sachs and Fogel, unpublished), such a clear distinction between these two properties was not found in the temperature tests.

The haemagglutinins from different tumours

Virus has been isolated from different polyoma induced tumours, and the antigenic similarity of the haemagglutinins determined by cross inhibition tests. The lines used were IL 1–6, and these consist of isolations from mouse parotid tumours, mouse mammary adenomas, a mouse mammary adenocarcinoma, and a rat kidney sarcoma. It was found that these virus isolates all gave cross haemagglutination inhibition, so that the haemagglutinins from different tumours have common antigens. Cross inhibition was also obtained with virus isolated from a C3HGr leukaemic mouse (Sachs *et al.*, 1959b). These results provide further evidence that more than one type of tumour may be produced by the same virus.

TABLE V.—*Ultraviolet Inactivation of Haemagglutinating Particles*

Time of irradiation (minutes)	Virus dilution 1 : 2	4	8	16	32	64	128	256	512	1024	2048	4096	8192	Saline control
None (control)	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	−	−	−
5	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	++	−	−	−
15	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	++	−	−	−
30	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	++	+	−	−
60	+++	+++	+++	+++	+++	+++	+++	+++	+++	++	−	−	−	−
70	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+	−	−	−
80	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+	−	−	−
90	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+	−	−	−
100	+++	+++	+++	+++	+++	+++	+++	+++	+++	+	−	−	−	−
110	+	++	++	+++	++	+++	++	++	++	+	−	−	−	−
120	−	+	++	+++	+++	+++	++	++	++	+	−	−	−	−
150	+	+++	++	+	+	++	++	+	−	+	−	−	−	−

TABLE VI.—*The Effect of Ultraviolet Irradiation on Haemagglutination and Infectivity*

Time of irradiation (minutes)	Original haemagglutination titre per 0·5 c.c. 1 :	Haemagglutination titre** after growth in tissue culture 1 : Period of growth (days) 5	8	11	Cytopathogenic effect on tissue culture Period of growth (days) 5	8	11	Haemagglutination inhibition titre after inoculation into 2-day-old mice. Time after inoculation (weeks) 2	4
None (control)	2048	1024	2048	2048	+	+	+	320	2,560
5	2048	N.T.	N.T.	N.T.	N.T.	N.T.	N.T.	320	10,240
15	2048	256	1024	2048	−	+	+	80	10,240
30	2048	32	512	256	−	−	+	160	10,240
60	1024	16	16	32	−	−	−	160	5,120
90	1024	32	16	8	−	−	−	80	10,240
120	512	32	16	16	−	−	−	160	5,120
150	128*	32	16	8	−	−	−	80	5,120
Normal tissue culture fluid	32	32	16	16	−	−	−	N.T.	N.T.

N.T. = Not tested. + = Positive. − = Negative. * Agglutination of + at dilution 1 : 2, 1 : 16 and 1 : 32. ** Titre per 0·5 c.c. in 4 c.c. of tissue culture fluid. End point for haemagglutination titres taken at ++.

TABLE VII.—*Heat Inactivation of Haemagglutinating Particles*

Treatment	Virus dilution 1: 2	4	8	16	32	64	128	256	512	1024	2048	4096	8192	Saline control
None (control)	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	–	–	–
56° C. for 30 minutes	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	–	–	–
62° C. ,, 30 ,,	+++	+++	+++	+++	+++	+++	+++	+++	+	+	–	–	–	–
64° C. ,, 15 ,,	+++	+++	+++	+++	+++	+++	+++	+++	+	–	–	–	–	–
64° C. ,, 30 ,,	+++	+++	+++	+++	+++	+++	+++	–	–	–	–	–	–	–
64° C. ,, 45 ,,	+++	+++	+++	+++	+++	+++	++	+	–	–	–	–	–	–
64° C. ,, 60 ,,	+++	+++	+++	+++	+++	+++	–	–	–	–	–	–	–	–
66° C. ,, 10 ,,	+++	+++	+++	+++	+++	+++	+++	++	+	–	–	–	–	–
66° C. ,, 15 ,,	+++	+++	+++	+++	+++	+++	+++	+	–	–	–	–	–	–
66° C. ,, 30 ,,	–	–	+	++	–	–	–	–	–	–	–	–	–	–
66° C. ,, 40 ,,	–	–	–	–	–	–	–	–	–	–	–	–	–	–
70° C. ,, 15 ,,	+++	++	–	–	–	–	–	–	–	–	–	–	–	–
70° C. ,, 30 ,,	–	–	–	–	–	–	–	–	–	–	–	–	–	–

TABLE VIII.—*The Effect of Temperature on Haemagglutination and Infectivity*

Treatment	Original haemagglutination titre per 0·5 c.c. 1:	Haemagglutination titre** after growth in tissue culture 1: Period of growth (days) 5	8	11	Cytopathogenic effect on tissue culture Period of growth (days) 5	8	11	Haemagglutination inhibition. titre after inoculation into 2-day-old mice 1: Time after inoculation (weeks) 2	4
None (control)	2048	1024	2048	2048	+	+	+	320	2560
56° C. for 30 minutes	2048	2048	2048	1024	+	+	+	320	2560
62° C. ,, 30 ,,	256	32	1024	1024	–	–	+	80	1280
64° C. ,, 15 ,,	256	64	512	1024	–	–	+	320	2560
64° C. ,, 30 ,,	128	32	64	512	–	–	?	320	1280
66° C. ,, 15 ,,	128	32	128	256	–	–	–	320	2560
66° C. ,, 30 ,,	16*	32	32	128	–	–	–	160	5120
70° C. ,, 15 ,,	4	16	32	64	–	–	–	160	5120
70° C. ,, 30 ,,	0	16	16	16	–	–	–	80	5120
Normal tissue culture fluid	32	32	16	16	–	–	–	N.T.	N.T.

* Agglutination of – at 1 : 2 and 1 : 4, and + at 1 : 8. + = Positive. – = Negative. N.T. = Not tested. ** Titre per 0·5 c.c. in 4 c.c. tissue culture fluid. End point for haemagglutination titres taken at + +.

Haemagglutination inhibitors in extracts of normal and tumour bearing animals

It has previously been found that extracts of tumours taken straight from the animal did not give haemagglutination, whereas high haemagglutination titres could be obtained from tumour cells in culture (Sachs *et al.*, 1959b). The absence of haemagglutination by extracts of tumours from the animal may be due either to a low haemagglutinin content or the existence of inhibitors. In the present study the latter possibility has been examined. Organs from normal mice, tumour bearing mice, and the tumours themselves, were homogenized with a motor driven homogenizer, centrifuged for 10 minutes at 9000 *g* in a Servall Angle Centrifuge, and the supernatant tested for haemagglutination inhibition. The tests were made in duplicate using supernatant unheated and heated for 30 minutes at 56° C., and the results compared with the effects of normal and immune serum. Three series of tests gave similar results and the data from one of these are given in Table IX.

The results show that inhibitors to a titre of 1 : 80 exist in organs from normal mice and that these are destroyed by heating at 56° C. for 30 minutes. Antibodies in immune serum, and the inhibitors (up to 1 : 40) in normal serum are not destroyed by this heating. In addition to the heat labile inhibitors, the extracts of organs from tumour bearing mice and from the tumours themselves also have high titres of antibodies, and both these, particularly the latter, could explain why extracts from tumours do not give haemagglutination. The presence of these inhibitors is also of significance in the actual isolation of viruses from tumours.

Haemagglutination inhibition antibodies and tumour formation in mice and hamsters

It has previously been shown that newborn mice inoculated with virus develop haemagglutination inhibition antibodies, and that there are high inhibition titres when palpable tumours are formed (Sachs *et al.*, 1959b). Further experiments were therefore carried out on haemagglutination inhibition antibodies and tumour formation in mice and hamsters. The newborn animals used were less than 24 hours old. Mice were inoculated subcutaneously with 0·05 c.c. and hamsters subcutaneously with 0·1 c.c. of virus suspension. Curves for inhibition antibody in three separate mice, tested at various intervals after virus inoculation into newborn, are shown in Fig. 2. In two of these with large tumours, the antibody titre increased up to the time of appearance of palpable tumours, and in the third, which had an unusual small slow growing tumour in the foot, the titre has continued to increase after the appearance of the tumour until it has so far reached 1 : 20,480. In the mice with the large tumours the same antibody titre was reached where the tumour appeared at 10 weeks and where it appeared at 23 weeks. Haemagglutination inhibition antibodies were therefore present in the mice during the entire period of growth after virus infection. Further data on this point are given in Table X, and these also show that the presence of palpable tumours is always associated with a high titre of inhibition antibodies.

In hamsters, although inhibition antibodies are also present after virus inoculation, the titres are lower in animals with palpable tumours than in the case in mice (Table X). Palpable tumours in hamsters develop earlier than in mice (Eddy *et al.*, 1958c). The earliest palpable polyoma induced tumours in our hamsters developed about 4 weeks after inoculation in contrast to about 9 weeks before the earliest palpable tumours are found in mice (Sachs *et al.*, 1959b).

TABLE IX.—*Haemagglutination Inhibition Tests for Inhibitors in Extracts from Normal and Tumour Bearing Mice*

			Extract or serum dilution 1 :													
			10	20	40	80	160	320	640	1280	2560	5120	10240	20480	40960	Saline control
Extracts from normal mice	Liver, lung kidney, and heart (pooled)	Unheated	–	–	–	–	+++	+++	+++	+++	+++	+++	+++	+++	+++.	+++
		Heated at 56° C. for 30 min.	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++.	+++
	Spleen, lymph-nodes, and thymus (pooled)	Unheated	H	H	+	+	++	+++	+++	+++	+++	+++	+++	+++	+++.	+++
		Heated at 56° C. for 30 min.	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++.	+++
Extracts from tumour-bearing mice	Liver, lung, kidney and heart (pooled)	Unheated	–	–	–	–	–	–	–	–	–	–	–	–	+++.	+++
		Heated at 56° C. for 30 min.	+++	+++	+++	+++	+++	++	+	–	–	–	–	–	+++.	+++
	Spleen, lymph-nodes and thymus (pooled)	Unheated	–	–	–	–	–	–	–	–	–	–	+++	+++	+++.	+++
		Heated at 56° C. for 30 min.	+++	+++	+++	++	+	–	–	–	++	+++	+++	+++	+++.	+++
Extracts from parotid tumours		Unheated	–	–	–	–	–	–	–	–	–	–	–	–	+++.	+++
		Heated at 56° C. for 30 min.	+++	++	+	–	–	–	–	–	–	–	++	+++	+++.	+++
Serum from normal mice		Unheated	–	–	+	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++.	+++
		Heated at 56° C. for 30 min.	–	–	+	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++.	+++
Serum from tumour-bearing mice		Unheated	–	–	–	–	–	–	–	–	–	–	–	–	+ .	+++
		Heated at 56° C. for 30 min.	–	–	–	–	–	–	–	–	–	–	–	–	++ .	+++

H = Haemolysis.

TABLE X.—*Haemagglutination Inhibition Titres at Various Intervals After Virus Inoculation into Mice and Hamsters*

	Mice			Hamsters		
Time after inoculation (weeks)	Titre 1 :	Palpable tumours	Age at time of inoculation (days)	Titre 1 :	Palpable tumours	Age at time of inoculation (days)
2	640*	–	2	2560	–	7
3	2560**	–	N.B.	2560	–	8
5	2560	–	N.B.	2560	+	2
5	5120	–	N.B.	5120	+	2
5	5120	–	N.B.	2560	+	2
7	5120	–	N.B.	2560	+	2
7	10240	–	N.B.	2560	+	2
9	10240	–	N.B.	2560	+	2
11	20480	–	N.B.	1280	+	5
11	40960	+	N.B.	2560	+	8
11	10240	+	N.B.	2560	+	3
12	10240	–	2	N.T.	N.T.	N.T.
12	40960	+	2	1280	+	3
13	> 20480	+	N.B.	640	+	5
13	40960	+	N.B.	640	+	5
14	20480	+	N.B.	640	+	3
15	20480	–	N.B.	2560	+	3
16	N.T.	N.T.	N.T.	640	+	3
20	> 5120	+	N.B.	N.T.	N.T.	N.T.
22	40960	+	N.B.	N.T.	N.T.	N.T.
22	5120	–	N.B.	N.T.	N.T.	N.T.

* Litter of 4 mice pooled. ** Litter of 7 mice pooled. N.B. = Newborn. N.T. = Not tested. + = Present. – = Absent. Each test represents the result from a different animal.

TABLE XI.—*Haemagglutination Inhibition Antibodies in AKR Mice*

		Antibody titre 1 :		
Number of mice	Age in weeks at 1st test	1st test	2nd test 4 weeks later	3rd test 8 weeks later
5	13	20	20	20
3	15	20	20	20
1	15	10	20	20
1	17	20	40	40
1	17	20	20	20
1	23	20	40	20
1	23	20	40	20
1	32	320	5120	N.T.
1	32	640	5120	2560
1	32	1280	2560	2560
1	32	1280	5120	2560
1	32	1280	5120	2560
1	32	1280	5120	2560
1	34	20	20	20
1	34	1280	N.T.	N.T.
1	35	20	10	20
1	35	20	N.T.	N.T.
1	36	10	N.T.	N.T.
1	36	2560	N.T.	N.T.

N.T. = Not tested.

Hamsters also have a different distribution of tumour types (Eddy *et al.*, 1958c), an observation that we have confirmed. The difference in inhibition antibodies between mice and hamsters does not seem to be due to the existence of an initially weak immune response in the hamster, since animals inoculated at 7 or 8 days of age (Table X) have antibody titres of 1 : 2560 after 2 or 3 weeks. The difference may be due to differences in cell virus relationships and the production of haemagglutinating particles. In addition hamsters may not produce titres higher than 1 : 5120, and there may be more antibody adsorption *in vivo* in hamsters than in mice. These possibilities are now being examined.

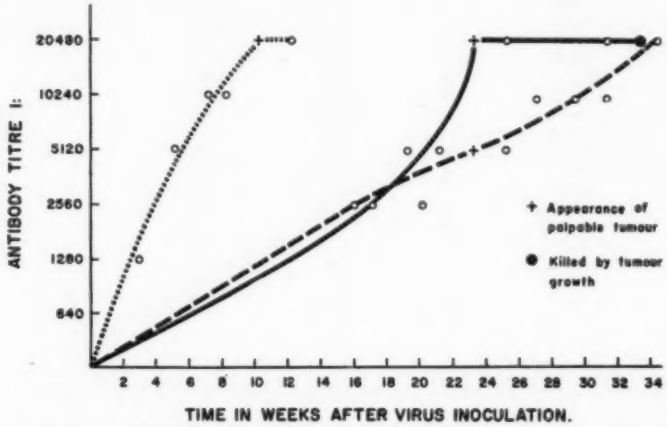

FIG. 2.—Haemagglutination inhibition antibody titres in relation to the appearance of palpable tumours in three mice inoculated as newborn with polyoma virus.

Haemagglutination inhibition antibodies in AKR mice

The existence of haemagglutination inhibition antibodies against polyoma virus in some AKR mice 32 weeks of age or older has already been reported (Sachs *et al.*, 1959b). These animals have now been further studied in order to obtain additional information on the existence of these antibodies in AKR, a strain of mice with a high incidence of spontaneous leukaemia. The results of two sets of tests, 4 and 8 weeks after the original titrations, are given in Table XI. These show that the animals with antibodies were also positive at the later tests.

All the mice have been kept as breeding pairs, and the 6 mice, 32 weeks old when first tested and found to have antibodies, are all litter mates belonging to the same family. In addition however, the 5 negative mice 13 weeks old at the first test, are litter mates derived from one of the pairs positive at 32 weeks. If the parents had been positive from the beginning, it may be assumed that their offspring should also be positive (Sachs *et al.*, 1959b, and unpublished), so that it appears that the mice positive at 32 weeks were negative at an earlier age. Four week old litters (7 animals tested) born from the negative mice 13 weeks old at the first test were also negative. Furthermore, both of the other positive mice shown in Table XI (one 34 and one 36 weeks old at the first test) have negative litter mates. These litter mates have been kept since birth in the same animal cage.

If the presence of inhibition antibodies in AKR were due merely to polyoma contamination in our laboratory, it would be expected that both members of the litter should have inhibition antibodies, since the presence of animals in the same cage seems to be the best means of ensuring contamination (Sachs *et al.*, 1959b, and unpublished). All AKR mice have been kept by us since their introduction in a different room from animals inoculated with polyoma virus. The existence of inhibition antibodies against polyoma in some AKR mice has also been observed by Rowe *et al.* (1958), who seem to interpret their results as due to contamination of the animals with polyoma.

DISCUSSION

The present studies have further shown the value of combining the *in vivo* with an *in vitro* approach in defining the properties of a mammalian tumour virus. The data on the haemagglutinating spectrum, and the rates of adsorption and elution, may serve as markers for the finding of mutants of the polyoma. Regarding the epidemiology of this virus, its high temperature stability would be a potent factor in determining its ability for survival and re-infection. In common with non-tumour forming viruses, infectivity is more sensitive than haemagglutination to ultraviolet inactivation, and although polyoma has a high resistance to ultraviolet inactivation, this could be a suitable method for virus inactivation and the maintenance of antigenicity. However for this latter purpose formalin has been found very satisfactory (Sachs and Fogel, unpublished).

The finding of haemagglutination inhibition antibodies in both mice and hamsters throughout their entire period of growth after virus infection, and the demonstration that tumour cells can continue to produce haemagglutinins (Sachs *et al.*, 1959b), makes it possible to determine the presence of this virus in tumours by testing the serum of animals for inhibition antibodies. An equivalent method has also been of value in studies on tumours induced in rabbits with papilloma virus (Rous, 1935).

It appears from the results obtained with AKR mice that some animals of this high leukaemic strain may develop inhibition antibodies against polyoma. Although it has been experimentally established that animal contamination with polyoma can take place (Sachs *et al.*, 1959b), the data presented, which include the observation that antibodies are found in older but not in young mice, make it unlikely that all the results with AKR are merely due to laboratory contamination. The possibility exists that virus activation may occur in AKR mice. Activation of a provirus may thus actually be demonstrable with a tumour virus, and further experiments in this direction are under way. In contrast to activation towards lytic action, i.e. a state that causes only cell destruction, as is found in lysogenic strains of bacteria, this would be activation to a stable cell virus relationship that under the appropriate conditions causes cell proliferation, i.e. the development of tumours.

SUMMARY

A study has been made of the haemagglutinating system of the polyoma virus with particular reference to the properties of the haemagglutinating particles, the formation of haemagglutination inhibition antibodies in relation to tumour formation, and the existence of such antibodies in AKR mice.

The polyoma can haemagglutinate in the cold (4° C.), the virus has a specific haemagglutination spectrum when tested with red blood cells from various species, and haemagglutination can take place at pH from 5·4–8·4.

Experiments on adsorption and elution have shown that the virus does not seem to possess a receptor destroying enzyme, that there is a rapid adsorption at 4° C., and a rapid elution at 37° C.

Sedimentation studies at 105,000 *g* have shown that haemagglutinins can sediment after 1 hour of centrifugation, but that even after 3 hours the supernatant still has some virus particles.

Ultraviolet irradiation experiments have demonstrated that in common with non-tumour forming viruses, infectivity is more sensitive than haemagglutination to inactivation with ultraviolet.

Temperature inactivation studies have shown a high stability of both infectivity and haemagglutination after 30 minutes at 56° C., and that there may still be some infective virus after 30 minutes at 70° C.

Virus isolation from polyoma induced parotid tumours, mammary adenomas and an adenocarcinoma in mice, and a kidney sarcoma in rats, gave cross haemagglutination inhibition, so that haemagglutinins from these different tumours have common antigens.

It was found that extracts of normal mouse organs possess haemagglutination inhibitors to a titre of 1 : 80, and that these inhibitors are destroyed after 30 minutes at 56° C. Extracts from organs of tumour bearing mice and from the tumours themselves contain inhibition antibodies in addition to these heat labile inhibitors.

Haemagglutination inhibition antibodies were shown to be present in animals throughout the entire period of growth after virus infection. In mice the presence of palpable tumours was always associated with a very high titre of inhibition antibodies, whereas hamsters with palpable tumours do not have such high titres.

Evidence has been presented to show that AKR mice may naturally develop haemagglutination inhibition antibodies against polyoma virus.

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BIBLIOGRAPHY

BUFFETT, R. F., COMMERFORD, S. L., FURTH, J., AND HUNTER, M. J.—(1958) *Proc. Soc. exp. Biol., N.Y.*, **99**, 401.

BURNET, F. M. AND STONE, J. D.—(1946) *Aust. J. exp. Biol. med. Sci.*, **24**, 1.

EDDY, B. E., ROWE, W. P., HARTLEY, J. W., STEWART, S. E. AND HUEBNER, R. J.—(1958*a*) *Virology*, **6**, 290.

Idem, STEWART, S. E. AND GRUBBS, G. E.—(1958*b*) *Proc. Soc. exp. Biol. N.Y.*, **99**, 289.

Idem, STEWART, S. E., YOUNG, R. AND MIDER, G. B.—(1958*c*) *J. nat. Cancer Inst.*, **20**, 747.

ELLERMAN, V. AND BANG. O.—(1908) *Zbl. Bakt.*, **46**, 595.

GROSS, L.—(1957) *Proc. Soc. exp. Biol. N.Y.*, **94**, 767.—(1958) *Cancer Res.*, **18**, 371.

HENLE, W. AND HENLE, G.—(1947) *J. exp. Med.*, **85**, 347.

HIRST, G. K.—(1942) *Ibid.*, **76**, 195; (1952) In 'Viral and Rickettsial Infections of Man,' 2nd Ed. (T. M. Rivers, editor). Philadelphia (J. B. Lippincott Co.), Chapter 4.

OBERLING, C. AND GUERIN, M.—(1954) *Advanc. Cancer Res.*, **2**, 353.

ROUS, P.—(1911) *J. exp. Med.*, **13**, 397.—(1935) *Harvey Lect.*, **31**, 74.

ROWE, W. P., HARTLEY, J. W., BRODSKY, I., HUEBNER, R. J. AND LAW, L. W.—(1958) *Nature, Lond.*, **182**, 1617.
SACHS, L., FOGEL, M. AND WINOCOUR, E.—(1959*a*) *Ibid.*, **183**, 663.
Idem, HELLER, E., MEDINA, D. AND KRIM, M.—(1959*b*) *Brit. J. Cancer*, **13**, 251.
SALK, J. E.—(1944) *J. Immunol.*, **49**, 87.
SMADEL, J. E.—(1948) In 'Viral and Rickettsial Infections of Man' (T. M. Rivers, editor). Philadelphia (J. B. Lippincott Co.), Chapter 3.
STEWARD, S. E., EDDY, B. E. AND BORGESE, N.—(1958) *J. nat. Cancer Inst.*, **20**, 1223.
WINOCOUR, E., AND SACHS, L.—(1959) *Virology*, **8** (in press).

EFFECT OF SARCOMA RD3 ON INTESTINAL ACTIVE ABSORPTION OF GLUCOSE AND L-HISTIDINE

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ONE of the important changes seen in a growing animal after the introduction of a transplantable tumour is the failure of the host to continue to gain weight at the same rate as control animals (Mider, Tesluk and Morton, 1948; Greenstein, 1954). In rats with a relatively large Walker Carcinoma 256 Bloor and Haven (1955) found that the intestine was considerably reduced in weight (both wet and dry) when compared with control animals of comparable size. Their findings suggested that when the tumour was about 50 per cent of the animal's total weight (carcass plus tumour) the intestinal weight was about three-quarters of that of comparable control animals, and they calculated that the ability of the intestine of their tumour-bearing rats to provide for adequate absorption was of the order of half the expected requirement. They therefore concluded that starvation and inadequate digestion and absorption were the bases of the tumour cachexia which they observed. In a recent consideration of the events occurring in the tumour-bearing animal Wiseman and Ghadially (1958) were also of the opinion that the intestine would suffer as a result of the rapid depletion of essential metabolites from the metabolic pool and that anorexia and cachexia would occur. The effectiveness of the small intestine of an animal as an absorbing organ depends, among other things, on the epithelial surface area of the intestine, and on the ability of the epithelial cells to transfer the products of digestion from the lumen of the intestine to the subepithelial space. In the experiments described in this paper we have investigated the ability of the small intestine of tumour-bearing animals to absorb glucose and L-histidine against their respective concentration gradients and have compared the results with those found in experiments with rats on a restricted diet. We found that the intestine of tumour-bearing rats could actively absorb glucose and L-histidine better than the intestine of control rats, and that in tumour-bearing rats the inadequacy of the intestine, as an absorbing organ, seems due to the decrease in the surface area of the epithelial lining (as shown in Fig. 1 and 2) rather than to interference with its ability for active absorption.

METHODS

Animals and diet

The animals used for all the experiments described in this paper were growing albino male rats of an inbred strain. The control animals (Group D) and those to be inoculated with tumour mince (Group A) were initially of similar weights of about 200 g. The animals which were to be fed a restricted diet (Groups B and C) were initially of such weights as to give final weights comparable with those of the tumour-bearing and control animals.

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All animals were kept individually in separating cages with free access to water. The food used in all the experiments was Diet 86, purchased from the North-Eastern Agricultural Cooperative Society Ltd., Bannermill Place, Aberdeen, and the theoretical composition is: soluble carbohydrate, 53·4 per cent; protein, 20·0 per cent; fat, 3·8 per cent; fibre, 3·3 per cent; ash, 5·2 per cent; moisture, 14·3 per cent.

All animals were inspected every day. Tumour-bearing rats (Group A) and control rats (Group D) were kept with a plentiful supply of food at all times. Of the animals on a restricted diet, those in Group B were fed 5 g. of food per rat per day for 5 days prior to their being killed, and those in Group C were fed 5 g. of food per rat per day for 9 days. The amount of food eaten by control rats (Group D) was of the order of 25 g./rat per day. The tumour-bearing rats (Group A) ate about 25 g./rat per day at the beginning of the experiment, but the consumption fell to about 20 g./rat per day by the time when they were killed.

Tumour-bearing animals with fairly large and still growing tumours were used at 14 ± 2 days after tumour inoculation. The control rats were used a similar length of time after the recording of their initial weights.

Tumour

The Sarcoma RD3 used in these experiments was originally induced by 1 : 2 : 5 : 6-dibenzanthracene injection into the right flank of an inbred strain of albino rats and has been successfully transplanted subcutaneously in this strain for about 22 years.

The animals were inoculated subcutaneously in the right flank with 0·2 ml. of a thick pasty suspension of tumour mince. The use of a thick paste instead of a watery suspension afforded a fairly accurate and simple method of administering equal amounts of tumour material to a group of animals, as problems due to the sedimenting out of cells during the course of injection did not arise. To each ml. of tumour paste was added approximately 100 mg. of streptomycin base and 50,000 units of crystalline penicillin, and the whole procedure was carried out with strict aseptic technique. There was never any macroscopic evidence of infection in any of the tumours induced by this method.

Preparation of sacs of intestine

The animals were killed by a blow on the head, the abdomen and thorax immediately opened, and the heart incised. The small intestine was then removed and everted, and sacs prepared as described by Wilson and Wiseman (1954). Six sacs were obtained from each small intestine, and their initial and final volumes were measured as described by Wiseman (1957). Occasionally the serosal volume of a sac decreased in volume: such sacs were discarded.

EXPERIMENTAL PROCEDURE

The sac, filled with a known volume (between 0·5 ml. and 1 ml.) of amino acid-glucose solution, was placed into a 150 ml. Erlenmeyer flask containing 20 ml. of the same solution as used for filling the sac. The air in the flask was then replaced with a gas mixture of 5 per cent CO_2 and 95 per cent O_2 and the flask tightly stoppered. The flask and its contents were then kept at 37°C and continuously shaken for 1 hour by the use of a Warburg bath (rate of shaking 80 oscil-

lations per minute, amplitude 5 cm.). At the end of the hour the sac was removed from the flask, its surface drained, and its fluid contents recovered and weighed. Samples of initial and final serosal and mucosal fluids were analyzed for amino acid and glucose concentrations. A short length of thread ligature left at one end of the sac greatly facilitates the removal of the sac from the flask.

Glucose and L-*histidine solutions.*—D-glucose of "Analar" grade (British Drug Houses Ltd., Poole) and L-histidine monohydrochloride of chemically pure grade were used without further purification. They were dissolved in bicarbonate saline (Krebs and Henseleit, 1932) to give a concentration of 0·3 per cent glucose and 2mM L-histidine monohydrochloride. The solution was gassed with 5 per cent CO_2 and 95 per cent O_2.

Chemical estimations.—Glucose was estimated by the colorimetric method of Nelson (1944). Histidine was estimated by the colorimetric method of Macpherson (1946).

Concentration ratios.—The concentration ratio referred to in the results is the ratio of the concentration of L-histidine or glucose in the serosal fluid (inside the sac of everted intestine) to the concentration of the respective chemical in the mucosal fluid (outside the sac).

Histological investigations.—Four rats (of weight 247 $\pm$ 28 g.) bearing Sarcoma RD3 (of weight 39 $\pm$ 5 g.) and four normal rats (of weight 249 $\pm$ 11 g.) were killed by a blow on the head. The pyloric part of the stomach, the pylorus and three inches of intestine were then removed in one continuous length. Approximately 5 ml. of 10 per cent formol-saline were passed through the lumen from the gastric end by means of a syringe so as to wash out the intestinal contents, and the terminal half-inch of intestine from each sample was then removed and fixed in 10 per cent formol-saline.

After four days' fixation in 10 per cent formol-saline each half-inch segment of intestine was trimmed and sliced carefully in the transverse plane so as to produce four or five small cylindrical segments. In order to avoid the occurrence of unequal shrinkage which might result from differences in processing technique, tissues from normal and tumour-bearing rats were treated identically during dehydration, clearing and impregnation with ester wax. Finally, the segments of intestine from each rat were blocked together, and sections 7 μ thick were prepared in the usual manner. The sections were stained with haematoxylin and eosin.

Standard deviations.—The figures shown throughout this paper are the means and standard deviations. Standard deviations were obtained using the formula for small samples when n was less than 30.

RESULTS

Macroscopic appearance of the small intestine

The intestine of the tumour-bearing rats appeared to be smaller in diameter than normal and was more translucent than that of the control animals. Intestinal

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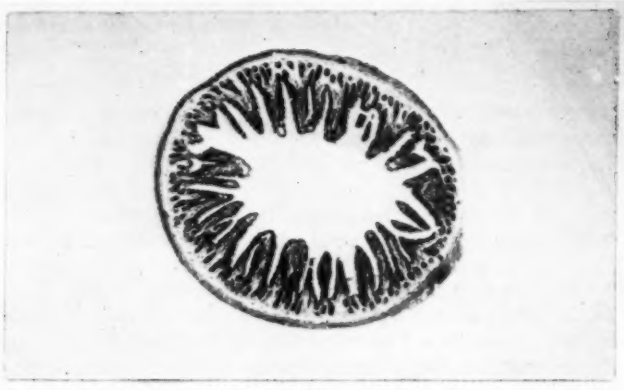
EXPLANATION OF PLATE

FIG. 1.—Rat bearing Sarcoma RD3: cross-section of intestine three inches distal to the pyloric sphincter. H. and E. × 25.

FIG. 2.—Normal rat: cross-section of intestine three inches distal to the pyloric sphincter. H. and E. ×25.



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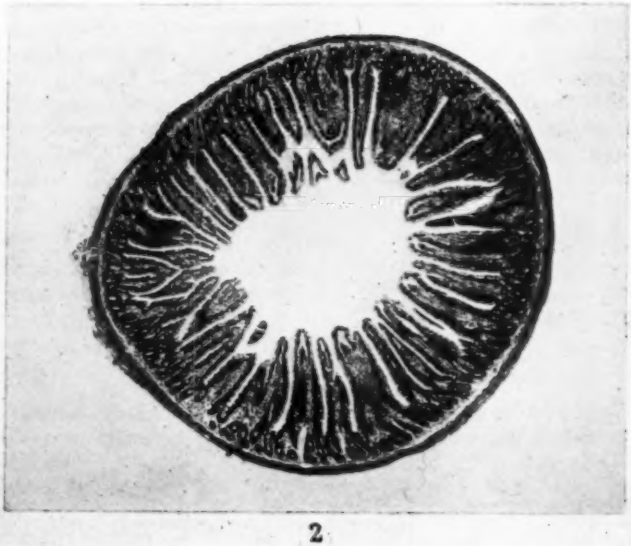

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villi are easily seen by the naked eye when a piece of everted intestine is filled with fluid, and it was noted that the mucosal surface of normal intestine was more luxuriantly covered with villi than that from tumour-bearing rats. There was never any obvious ulceration or necrosis of the mucosa. The changes seen in the intestine of rats fed a restricted diet for 9 days (Group C) were substantially the same as those in the tumour-bearing animals.

Microscopic appearance of the small intestine

The histological picture seen in the intestine of the tumour-bearing rat (Fig. 1) is one of generalized atrophy of all the coats of the intestine as compared with that of a control animal (Fig. 2). It will be observed that the intestine of the tumour-bearing animal is much smaller in diameter, and that the villi are reduced in number and size. A reduction in the size of the epithelial cells lining the villi as compared with normal could also be seen. There was no evidence of necrosis, ulceration or inflammatory changes. Fig. 1 shows only an apparent slight reduction in the thickness of the submucous and muscular coats in the intestine of the tumour-bearing animal as compared with that of the control animal shown in Fig. 2, but when the appearance is intepreted in conjunction with the reduced diameter of the intestine of the tumour-bearing animal there can be no doubt that there is considerable atrophy of the submucosal and muscular coats of the intestine of the tumour-bearing animal. The degree of atrophy varied within the group of tumour-bearing animals, but in each case it was sufficiently well-marked to distinguish the intestine of a tumour-bearing animal from that of a control animal. Fig. 1 and 2 were taken from representative members of each group and illustrate the average and not the maximum degree of change observed in the tumour-bearing animals. The changes seen in the intestine of rats fed a restricted diet for 9 days were substantially the same as those in the tumour-bearing animals.

Active absorption of glucose and L-histidine

Table I shows the concentration ratios which were produced by sacs of small intestine from rats bearing sarcoma RD3 (Group A), rats on a restricted diet (Groups B and C), and control rats (Group D). It will be seen that the intestine of the animals in Group A was able to produce, within the experimental period

TABLE I.—*Active Absorption of Glucose and L-histidine by Sacs of Everted Small Intestine from Rats Bearing Sarcoma RD3, Rats on Restricted Diets, and Control Rats*

Group	Number of animals	Animal weights (g.)		Number of sacs	Concn. ratios developed (serosal concn./mucosal concn.)	
		Initial	Final		L-histidine	Glucose
A. Sarcoma RD3	8	206±12	244±27 (including tumour wt. of 46±16)	46	2·89±0·62	2·63±0·45
B. restricted diet for 5 days	6	269±10	235±8	34	2·74±0·82	2·38±0·39
C. restricted diet for 9 days	6	285±12	227±8	32	3·17±0·75	2·63±0·35
D. controls	6	214±23	258±16	35	1·81±0·31	1·440±·32

of one hour, mean concentration ratios of 2·89 and 2·63 for histidine and glucose respectively, whereas the mean concentration ratios produced by the intestine from control rats were 1·81 for histidine and 1·44 for glucose. A comparison of the concentration ratios for Group A with those produced by animals on a restricted diet shows that the Group A animals gave results substantially the same as those in Group C.

Water uptake by the sacs

The sacs of everted intestine from the tumour-bearing rats showed the same ability to absorb water as did the sacs from control animals. The serosal volumes of the former increased by 34 ± 19 per cent and the serosal volumes of the latter increased by 30 ± 24 per cent. The serosal volumes of the sacs from animals on a restricted diet for 5 days increased by 45 ± 27 per cent and those on a restricted diet for 9 days increased by 37 ± 22 per cent.

DISCUSSION

The macroscopic and microscopic changes found in the intestine of our tumour-bearing rats support Bloor and Haven's (1955) view for the cause of cachexia and death in such animals, namely "the amount of intestinal tissue is insufficient to support life and growth in the face of the competition of the tumour." Our finding of an increased ability for active absorption by the tumour-bearing animals suggests that some compensatory change occurs in an intestine from an animal whose caloric intake is insufficient, and possibly for some time such increased absorptive ability may help overcome the decrease in absorbing surface. It is interesting to note that our non tumour-bearing animals on a restricted diet also showed an increase in absorptive ability at the same time as generalized intestinal atrophy was occurring. The increased concentration ratios produced by sacs from experimental animals (Groups A, B and C) cannot be explained by a decreased water absorption, as there was no difference in the uptake of water by those sacs when compared with controls. Although the tumour-bearing rats ate considerably more than those on a restricted diet, the intestines of the rats in both groups were remarkably similar in macroscopic and microscopic appearance, and also in their ability to absorb actively glucose and L-histidine. The food consumed by the tumour-bearing rats (up to the time of being killed) was not much less than that eaten by the control animals on an *ad lib* diet, and yet the intestine of the tumour-bearing rat had an appearance, and absorbing capacity, similar to that of an intestine from an animal which had been on a severely restricted calorie intake (Group C). The demands of the rapidly growing tumour for metabolites therefore reduces the intestinal tissue to the same condition as the intestine from an animal on a bare subsistence diet, and we agree with Bloor and Haven (1955) that with continued growth of the tumour the intestine is reduced to a condition where, despite any enhanced ability for absorption which it may have acquired, the actual amount of intestinal tissue becomes inadequate for the needs of the normal plus tumour tissue.

SUMMARY

The ability to absorb glucose and L-histidine against their respective concentration gradients was investigated in rats bearing Sarcoma RD3, in rats on

restricted diets, and in control rats. The method used was the sac of everted small intestine as described by Wilson and Wiseman (1954).

It was found that the small intestine of the tumour-bearing animals and of the animals on restricted diets had an enhanced ability for active absorption as compared with control animals. This finding was in contrast to the wasting seen in the intestine of the tumour-bearing animals and those on a restricted diet.

It is suggested that any inadequacy occurring in the absorptive capacity of a tumour-bearing animal's small intestine is due to a decrease of tissue rather than to interference with its ability for active absorption.

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REFERENCES

BLOOR, W. R. AND HAVEN, F. L.—(1955) *Cancer Res.*, **15**, 173.
GREENSTEIN, J. P.—(1954) ' Biochemistry of Cancer '. New York (Academic Press).
KREBS, H. A. AND HENSELEIT, K.—(1932) *Z. physiol. Chem.*, **210**, 33.
MACPHERSON, H. T.—(1946) *Biochem. J.*, **40**, 470.
MIDER, G. B., TESLUK, H. AND MORTON, J. J.—(1948) *Acta Un. int. Cancr.*, **6**, 409.
NELSON, N.—(1944) *J. biol. Chem.*, **153**, 375.
WILSON, T. H. AND WISEMAN, G.—(1954) *J. Physiol.*, **123**, 116.
WISEMAN, G.—(1957) *Ibid.*, **136**, 203.
Idem AND GHADIALLY, F. N.—(1958) *Brit. med. J.*, ii, 18.

THE DOSE-RESPONSE RELATIONSHIP OF THE EHRLICH ASCITES TUMOUR

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THE work described in this paper was carried out as a preliminary study on the " viability " of Ehrlich ascites tumour cells. Several tests have been described, using stains, which purport to distinguish " viable " from " non-viable " cells. However Hoskins, Meynell and Sanders (1956), using the ascites form of the Krebs-2 carcinoma, have pointed out that the results of such tests are not clearly related to the ability of tumours to grow in susceptible hosts.

It is plain that there are two factors involved in the production of tumours in animals following the inoculation of tumour cells. The first is the " viability " of the inoculated cells and the second the " susceptibility " of the host. Serial dilutions of a suspension of tumour cells may be inoculated into mice and the proportion developing tumours recorded and plotted against the dose or number of tumour cells inoculated. The resulting dose-response curve may provide information concerning the viability of the cells and the susceptibility of the host animals.

The study of dose-response relationships has received most attention in the field of pharmacology where the material comprising the dose is not particulate in the ordinary sense and can theoretically be varied infinitely. In the application of such studies to viruses, recently reviewed by Isaacs (1957), the dose consists of a number of virus particles and results have indicated that the appropriate mathematical distribution is often provided by the Poisson or exponential model. The purpose of some of the work on viruses has been to determine what proportion of particles in a virus suspension are viable and whether a single viable particle can initiate infection. A difficulty frequently encountered in this field has been to obtain a reliable method of counting the total (viable and non-viable) number of particles in a particular preparation. This difficulty does not arise with suspensions of ascites tumour cells as these are easily counted in a haemocytometer. Therefore it seemed that a study of the dose-response relationship of an ascites tumour, along the lines described by virus workers, could provide valuable information concerning the viability of its constituent cells and should, therefore, be investigated.

This paper describes the results obtained from the intraperitoneal inoculation of serial dilutions of Ehrlich ascites tumour cells into a strain of white mice.

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For reasons that will become clear it was found that the methods used here were not suitable for the direct investigation of the viability of our tumour cells, but this result is of interest in itself, as is other information revealed by our experiments.

Theoretical introduction

The theoretical considerations which follow are based on the assumption that a dose-response curve is obtained from observing proportions of animals responding with tumour following the inoculation of serial dilutions of a suspension of tumour cells. For the sake of simplicity in this paper animals developing tumours following the inoculation of tumour cells will be described as having been "infected". The term "viability" is used in the sense of the ability of a cell to infect a completely susceptible host. It will be assumed that tumour cells in suspension have a Poisson distribution where "a" is the dilution and "m" is the concentration before dilution.

There appear to be several possible ways in which tumour cells and host animals may interact:

(a) All tumour cells in a suspension may be viable and may be "autonomous" in the sense that their growth is entirely independent of host factors. The animals may, in fact, be completely susceptible. If this is so, the proportion, P, of animals developing tumours in dilution, a, of a suspension containing m tumour cells

$$= 1 - e^{-am}$$

where e^{-am} is the first term of the Poisson series.

Since we may easily estimate the total number of cells inoculated by counting them, it would be necessary only to compare the observed results with those calculated to test the validity of this hypothesis. The shape and slope of the dose response curve will be that of the "one-particle" curve described by Isaacs (1957) and its position will be determined by m, the number of cells in the original suspension.

(b) Only a proportion of the cells in the original suspension may be viable although the hosts may be completely susceptible.

Let $c = am$ be the number of cells in dilution a. If p is the proportion of viable cells, then

$$P = 1 - e^{-cp}$$

The dose response curve would be exactly the same as in (a) except that it would be shifted to the right. Knowing the total number of cells, it would be an easy matter to obtain a value for p, provided we knew that the hosts were completely susceptible.

(c) Hosts may not be completely susceptible, but their susceptibility may be uniform, when

$$P = 1 - e^{-cph}$$

where h = the probability that a viable cell will infect a particular host. In this case the value of h is the same for all mice. The dose-response curve would be similar to that of (b) and a value for h could only be obtained if p were known and *vice versa*.

(d) More than one viable tumour cell may be required to initiate a tumour, but the same number of viable cells will initiate tumours in all animals. Let

g = the number of viable tumour cells producing tumours in all animals, then

$$P = 1 - e^{-cp}\,[1 + cp + (cp)^2/2! + \ldots + (cp)^{g-1}/(g-1)!]$$

In this case the slope of the dose response curve would be greater than that discussed in (a), (b), and (c), and would provide an estimate for g. Knowing this we could obtain an estimate for p using our knowledge of the total number of cells inoculated.

(e) The susceptibility of the hosts may be variable and

$$P = \int_0^1 (1 - e^{-cph})\, f(hp)\, d(hp)$$

It this were so it would not be possible to make predictions concerning the dose-response curve except that in general the slope would be less than those for the foregoing possibilities. If we could show that the slope were less than the exponential curves described in (a), (b), and (c) it would be a clear indication that host susceptibility was variable from animal to animal. Under these circumstance it would be impossible to reach firm conclusions concerning the viability of the cells.

(f) A final possibility exists where there may be a combination of the situations described in (d) and (e) where the susceptibility of the hosts may be variable and more than one viable tumour cell is required to produce a tumour in completely susceptible hosts. In this case

$$P = \int_0^1 \{1 - e^{-chp}[1 + chp + (chp)^2/2! + \ldots + (chp)^{g-1}/(g-1)!]\}\, f(hp)\, d(hp)$$

Another way of expressing this situation would be to imagine that the production of tumours occurs in two stages. The first stage consists of the implantation of viable cells and the second stage their growth or progression. Whether or not the first stage occurred could be governed by the susceptibility of the animals. If we could detect this stage the probability of its occurrence would be governed by the equation in (e). However, subsequent growth and progression into a tumour would occur only when g or more cells had established themselves. This distribution, however, could not be distinguished from that described in (e).

It follows from the above considerations that it is possible to distinguish susceptibility of hosts from viability of cells only if the dose-response curves are those described in (a) or (d).

If the dose-response curve is similar to those in (b), (c), (e), or (f), it will be impossible to obtain estimates for either susceptibility or viability unless one or other of them is known, as is pointed out by Hoskins, Meynell and Sanders (1956). However, it would be possible to obtain an estimate of a quantity α (where $\alpha = hp$) which is the probability that a cell drawn at random from a preparation will infect a particular host. If host susceptibility is uniform for all mice, α would be represented by single value as in (b) and (c), whereas if host-susceptibility is variable from animal to animal, as in (e) or (f) α would follow a distribution. Hence, α describes the interaction between a particular group of animals and a particular preparation of tumour cells. To avoid ambiguity we can speak of the quantity α as the " sensitivity " of a particular host-cell system remembering that

it is the product of the " viability " of the cells and the " susceptibility " of the hosts.

In examining a dose-response curve of the sort discussed here, the first step would be to determine whether it departed from the exponential model. In this work we have applied the method of probit analysis as described by Finney (1952). Armitage and Spicer (1956) have discussed methods for detecting departures from exponentiality and suggest that probit analysis would be suitable for this purpose and, as our results show, we have found it so.

MATERIALS AND METHODS

Six experiments were carried out in this series, spread out over the greater part of two consecutive months.

Animals.—The Institute strain of pen-bred white mice were used at an age of between 3 and 4 weeks, within one week of having been weaned. The day before each experiment, mice obtained from the previous week's weaning were distributed at random among five groups. Four of the groups were distinguished by being painted different colours and the remaining group left uncoloured. On the following day (the morning of the day of inoculation) each group was distributed at random, with the restriction that there was only one mouse from each group in each cage. Twenty mice were used for each dilution of tumour cell suspension in each experiment. Hence four cages of mice were used for each dilution in each experiment.

Ascites tumour.—The hyperdiploid line of the Ehrlich ascites tumour was received from Dr. Hauschka in its 113th ascites generation. In this Institute it has, except for the first passage, been passed at weekly intervals. The experiments were carried out on material which had been through at least 35 serial passages in the Institute strain of mice.

Preparation of tumour cell suspensions.—On the day of each experiment and within one hour of inoculation, mice inoculated with tumour cells between 5 and 7 days previously were killed, the ascitic fluid removed aseptically and placed in 1 oz. screw-capped bottles. The bottles were placed on a mixer consisting of a wheel inclined at 45 degrees to the horizontal and rotated at 25 to 30 r.p.m. for five minutes. After a preliminary count of the tumour cells, some fluid was removed and diluted so that a final count could be carried out conveniently in a haemocytometer. At least 200 tumour cells were counted on each occasion.

After the tumour cell concentration had been determined, sufficient Earle's solution was added so that the concentration of tumour cells was 2 million in each 0·2 ml. Dilutions for inoculation were prepared from this suspension. In the first three experiments the suspension containing 2 million cells per 0·2 ml. and six fourfold dilutions of it (1 : 4 to 1 : 4,096) were used for inoculation. In the last three experiments seven twofold dilutions (1 : 16 to 1 : 1,024) of the suspension were used.

In the first experiment (Experiment 70) the dilutions were made just before each one was required for inoculation – thus, the original suspension was inoculated and then a 1 : 4 dilution was made and inoculated and then a 1 : 16 dilution prepared and so on to 1 : 4,096. In the remaining five experiments all dilutions were prepared before any inoculations were carried out.

The conduct of inoculation.—In the first experiment the mice were inoculated

in order from the lowest to the highest dilution. In the remaining five experiments the order in which the cages were inoculated was assigned at random.

A tuberculin syringe (1·0 ml.) full of the appropriate dilution was used for each cage, each mouse received 0·2 ml. of suspension intraperitoneally by inoculation through the right posterior quadrant of the abdominal wall. A separate syringe was used for each dilution. The mice in each cage were always inoculated in the same order, being identified by their colours. Hence, it was possible to identify mice that had received the first, second, third, etc., doses in the syringe.

Observations of mice.—The mice were examined daily for deaths. Three times a week they were examined for abdominal swelling, this was recorded as slight, moderate, or marked. Those mice showing progressive abdominal enlargement ending in sickness and death were regarded as having " definite ascites " tumours (" definite ascites " or *DA*) and no post-mortems were carried out on them.

Other mice dying during the observation period, including those with doubtful abdominal distension were subjected to post-mortem. Of these, those without tumour visible to the naked eye were excluded from the subsequent analysis. Those with tumour visible to the naked eye were recorded as " other tumour " (*OT*) mice, except for those with tumour and about 1 ml. or more of peritoneal fluid which were recorded as " equivocal ascites " (*EA*) mice. Since mice had been dead for some unknown number of hours before post-mortem examination, if was often not possible to decide for certain whether small amounts of peritoneal fluid were the result of putrefaction or not. In some mice putrefaction was too advanced for an adequate post-mortem examination and these were excluded from our final analysis.

The mice were observed for a period of 60 days after which all the survivors were killed and post-mortem examinations carried out on them. Those without tumour were recorded as " non-tumour " (*NT*) mice and included in the analysis. Those with tumour were recorded as " other tumour " (*OT*) or " equivocal ascites " (*EA*) as described above and included in the analysis.

Mathematical treatment.—The results of total tumour incidence

$$[(DA + OT + EA)/(DA + OT + EA + NT)]$$

in each experiment (Table I) were subjected separately to probit analysis according to the method of Finney (1952) and subsequently combined. Probits were plotted against the logarithm to the base 2 of the dilution of the original suspension. The proportions of definite ascites mice, while neglecting all other tumour bearing mice $[DA/(DA + NT)]$ (Tables I, II) from all experiments were pooled and then subjected to probit analysis. Finally, the proportions of definite ascites mice were considered (Tables I, II) while all other tumour bearing mice and those without tumours were combined as the non-responding group $[DA/(DA + OT + EA + NT)]$ (Table II). Note that $(DA + OT + EA + NT) =$ " Total mice ", i.e. all mice included in the final analysis.

RESULTS

In the six experiments described here it would have been possible to have obtained observations on a total of 840 mice. However, of these, 32 were dead before inoculation or were missing at some time during the course of the experiments. In the early stages 97 mice died, mostly within the first week of inoculation,

TABLE I.—*Distribution of Tumour Bearing Mice according to Dose of Ehrlich Ascites Tumour Cells and Experiments*

Dilution $\log_2$	Experiment numbers						Totals: Definite ascites	Totals: Other + equivocal tumours	Totals: No tumour	Grand total
	70	73	74	82	85	90				
+1	19, 0, 0	9, 3, 0	18, 0, 0	*	*	*	46	3	0	49
−1	16, 0, 0	10, 3^1, 2	18, 0, 0	*	*	*	44	3(2)	2	49
−3	12, 1^1, 0	12, 0, 0	13, 3, 0	12, 2^1, 0	10, 3^1, 0	6, 3^1, 0	65	12(4)	0	77
−4	*	*	*	10, 4^2, 0	9, 5, 0	10, 7^1, 0	29	16(3)	0	45
−5	9, 6, 0	5, 4, 4	11, 3, 4	7, 4, 0	8, 6, 2	8, 2, 2	48	25	12	85
−6	*	*	*	4, 7, 2	6, 4, 1	6, 6, 2	16	17	5	38
−7	7, 4^1, 4	4, 5^2, 3	9, 4^1, 3	8, 4, 3	7, 6, 2	11, 3, 3	46	26(4)	18	90
−8	*	*	*	9, 1, 2	7, 6^1, 2	10, 6, 3	26	13(1)	7	46
−9	10, 2, 8	10, 2, 5	6, 3, 6	5, 0, 0	10, 2, 3	7, 2^1, 7	48	11(1)	29	88
−11	4, 1, 12	8, 0, 8	6, 2, 6	*	*	*	18	3	26	47
						Totals	386	129	99	614

In each group of three figures

(1) The first is the number of "definite ascites" tumours (*DA*).

(2) The second is the sum of the "other tumours" (*OT*) and "equivocal ascites" (*EA*)—the superscripts indicate the number of *EA* included.

(3) The third are those surviving 60 days without tumour (*NT*).

The figures in brackets among the "Totals" indicate the number of *EA* included in the total preceding it.

* = not examined.

TABLE II.—*Percentage Incidence of Mice with Tumours Following Inoculation of Ehrlich Ascites Tumour Cells: Ratio of Ascites to Non-Ascites Tumours*

Dilution $\log_2$	Per cent tumour incidence: $\frac{(DA+OT+EA)}{\text{Total mice}}\times 100$	Definite ascites tumours with: 1. Non-tumour $\frac{DA}{DA+NT}\times 100$	Definite ascites tumours with: 2. All other mice $\frac{DA\times 100}{\text{Total mice}}$	Ratio Definite ascites/Other tumours $\frac{DA}{OT}$
1	100	100	94	15·3
−1	96	96	90	44·0
−3	100	100	84	8·1
−4	100	100	64	2·2
−5	86	80	57	1·9
−6	87	76	42	0·9
−7	80	72	51	2.1
−8	85	79	57	2·2
−9	67	62	55	4·8
−11	45	41	38	6·0

The proportions shown in this table are obtained from the figures shown in Table I.

apparently from non-specific causes. During the later stages 159 mice died of which 79 were subjected to adequate post-mortems and of these 66 showed evidence of tumour growth and included those with equivocal ascites tumours. In addition, 386 mice showed progressive characteristic abdominal distension followed by death and were designated definite ascites tumour mice. In the lowest dilution progressive abdominal swelling began from 10 to 21 days after inoculation and in the highest dilution from 22 to 40 days after inoculation. Deaths

of these mice occurred 14 to 32 days after inoculation in the lowest dilution and 30 to 49 days after in the highest. The number of mice surviving the observation period of 60 days was 166 of which 162 was subjected to post-mortem. Of these 63 had evidence of tumour growth and 99 were free of it. Thus, a total of 614 mice provided our observations and the distribution of these is shown in Table I.

Among those mice coming to post-mortem, three types of tumour were observed. Inoculation tumours were those in the abdominal wall at the site of inoculation. "Soft" tumours were similar to those described for the ascites form of Sarcoma 37 (Warner, Kroeker and Lederman, 1957) and consisted of friable whitish tumour material in the substance of tissues. "Hard" tumours were well-knit firm tumours similar to those obtained by subcutaneous inoculation.

Soft tumours tended to occur relatively soon after inoculation during the period when mice inoculated with the same number of cells were developing and dying from definite ascites tumours. Hard tumours tended to occur later than soft tumours.

Among those 66 mice dying with tumour during the period of observation approximately half had hard tumour and half soft tumour. Five mice had both types of tumour simultaneously. Twenty-three of these mice had inoculation tumours in addition to the other tumours mentioned.

Of those mice surviving the observation period of 60 days and revealing tumour at post-mortem, 49 had hard tumour and two of these had soft tumour in addition. In 14 mice the only evidence of tumour was at the site of inoculation.

Nearly one half of all the hard tumours occurred in the mesentery of the small intestine and approximately one quarter in the region of the pancreas. The remainder were more or less equally distributed between the regions of the kidney, the internal genital organs and the parietal peritoneum.

Of 34 soft tumours, 16 were in the pancreatic region only, 13 in both the pancreatic and genital regions and two in the genital region only. The remaining three were in the region of the kidney.

Fluid of approximately 1·0 ml. or more was observed in 15 mice, including two that had survived the observation period. Soft tumour was present in 13 of the mice with fluid and was accompanied by hard tumour in two instances. In the remaining two mice fluid was accompanied by hard tumour only.

There was no evidence that the tumour incidence in those mice inoculated with the first or any other dose in a syringe differed from one another.

The results of the probit analysis, based on the proportions of animals responding with any type of tumour ("Total tumours") show that all six experiments, carried out at different times, may be represented by a single curve and that any departures from it may be accounted for by chance alone as is shown in the Table of Analysis of χ^2.

The slope of the curve is approximately 0·2 and, by being significantly different from 2, indicates departure from exponentiality (Armitage and Spicer, 1956). From these results it is estimated that the number of cells which will produce tumours in 50 per cent of animals is approximately 850.

The pooled results of all experiments are shown in Fig. 1 and Table II. The smooth line fitted to the points in circles for total tumour incidence is obtained from the probit regression equation

$$Y = 7{\cdot}33 + 0{\cdot}21\,x$$

The exponential curve showing the best fit (Haldane, 1939) to our data is shown in Fig. 1 as an interrupted line, and it is clear that the observed distribution of total tumour incidence differs markedly from it.

An analysis of those responding with definite ascites tumours and those failing to respond at all (i.e. excluding those in the " Other Tumours " column in Table I) provides a curve of shape and slope similar to that obtained when tumours of all types are considered. However, the curve is shifted slightly to the right. The points are not plotted in Fig. 1 for the sake of clarity.

The points in Fig. 1 indicated by solid circles are obtained by regarding " Definite Ascites " only as the response and including among the non-responders

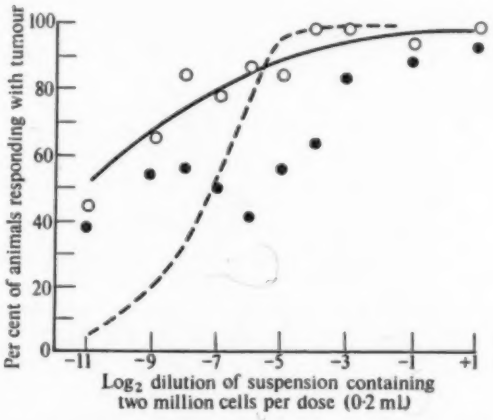

FIG. 1.—Shows the percentage of animals responding with tumours at graded doses of tumour cell suspension.

○ = Total tumours [$(DA+OT+EA)$/Total mice]
● = Definite ascites tumours as a proportion of all mice [DA/Total mice].
———— = Curve obtained from probit-log dose regression equation.
– – – – – = Curve obtained from Haldane's (1939) maximum likelihood estimation of the exponential.

those listed as " Other Tumours ", " No Tumours ", and " Equivocal Ascites." The distribution is considered here firstly to see if it provided evidence that the ascites response is a completely separate phenomenon from other types of tumour growth. Secondly, the distribution is somewhat irregular, there being a dip in the centre. However, the proportion of tumours occurring at the −6 dilution is not significantly different from the proportion of tumours in the pooled results of the −7, −8, and −9 dilutions ($\chi^2_{[1]} = 1{\cdot}3$, $p = 0{\cdot}26$).

Table II shows the ratios of " definite ascites " tumours to " other tumours " for each dilution of tumour cell suspension. For the purpose of obtaining these ratios we have neglected entirely the " Equivocal Ascites " tumours. The ratios are plotted in Fig. 2 and a curve drawn through them by eye. It will be seen that the ratio is high in the lowest dilutions and falls to a minimum at dilutions −4, −5, and −6 which are logarithms to the base 2 of the dilution. Thereafter the ratio shows a progressive increase as the cells inoculated decrease.

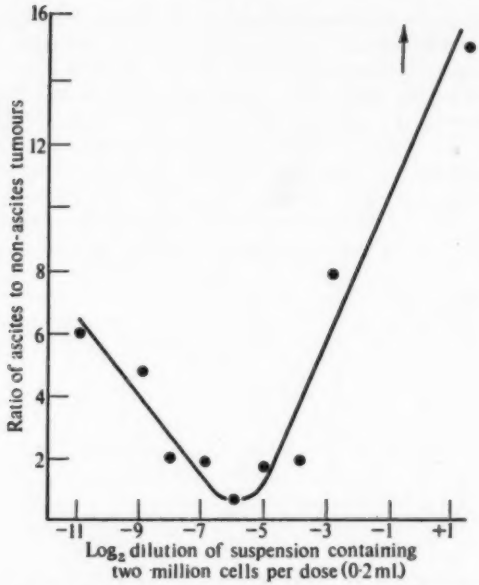

FIG. 2.—Shows the ratio of definite ascites tumours to other tumours (*DA/OT*) at graded doses of tumour cell suspension. The curve is drawn by eye.

TABLE III.—*Analysis of* χ^2

	Degrees of freedom	Sum of squares	Mean square
Parallelism of regressions	5	8·374	1·675
Residual heterogeneity	30	24·575	0·819
Total	35	32·949	—

DISCUSSION

The results of the present investigation has shown that the susceptibilities of the animals used in these experiments are variable and are normally distributed when plotted against the logarithm of the number of cells inoculated. Hence, in our animals, the cells of the Ehrlich ascites tumour cannot be called "autonomous" in the sense that they are entirely independent of host-factors. In consequence of these findings, no conclusions concerning the viability of the ascites tumour cells can be drawn since it is indistinguishable from host susceptibility. However, we can, as we have shown in our introduction, consider the quantity $\alpha = hp$ which is the probability of infection of a host by a single cell and which we have called the "sensitivity" of the host-cell system. This we see would be variable from host to host and thus is covered by sections (e) and (f) in the theoretical introduction.

Firstly, dealing with the situation described in (e) we find that the distribution of the sensitivity can be deduced from the dose-response curve by following the

"alternative approach" given by Armitage and Spicer (1956). This is most appropriate here because, as we shall see, the sensitivity distribution must be approximately log normal.

The probability that a mean dose of c cells will infect a host in a system of sensitivity α is—

$$1 - e^{-c\alpha} \tag{1}$$

If the sensitivities are distributed with probability density $f(\alpha)$, then the probability of infection of a host selected at random is

$$P = \int_0^1 (1 - e^{-c\alpha}) f(\alpha)\, d\alpha$$

$$= \int_{-\infty}^{0} (1 - e^{-e^{x+y}})\, \varphi(y)\, dy \tag{2}$$

where $x = \log c$, $y = \log \alpha$, $\varphi(y)\, dy = f(\alpha)\, d\alpha$

Define $\varphi(y) = 0$ for $y > 0$ and

$$P = \int_{-\infty}^{\infty} (1 - e^{-e^{x+y}})\, \varphi(y)\, dy \tag{3}$$

One can see that this is the distribution function of a variate

$$x = z - y \tag{4}$$

where z is a variate distributed independently of y with a distribution function

$$1 - e^{-e^{z}} \tag{5}$$

From (4) and independence of z and y, we infer that the mean $E[x]$ and variance $V(x)$ are given by

$$E[x] = E[z] - E[y]$$
$$V[x] = V[z] + V[y]$$

hence

$$E[y] = E[z] - E[x]$$
$$V[y] = V(x) - V(z)$$

Irwin (1942) has studied the distribution of z and shown that

$$E[z] = -\gamma = -0{\cdot}577$$
$$V(z) = \pi^2/6 = 1{\cdot}645$$

From the fitted probit line we have the estimates for

$$E[x] : \log (2 \times 10^6) - 11{\cdot}2 \log_e 2 = 6{\cdot}745$$
$$V(x) : 23{\cdot}15 (\log_e 2)^2 = 11{\cdot}123$$

Hence the mean and variance of the distribution of the log sensitivities y are estimated to be

$$E[y] : -7{\cdot}322$$
$$V(y) : \quad 9{\cdot}478 \qquad S.D. \quad 3{\cdot}079$$

Since the probit line fits the data, x is approximately normally distributed. z is not too far from normal either, and in any case, $V(z)$ is only a small component of $V(x)$. Thus y must be approximately normal, i.e. we have shown that the distribution of the log sensitivities is approximately normal, as mentioned earlier.

Our results may be summarized by plotting the distribution of sensitivities (Fig. 3). For convenience we use a scale of common logarithms, i.e. we plot

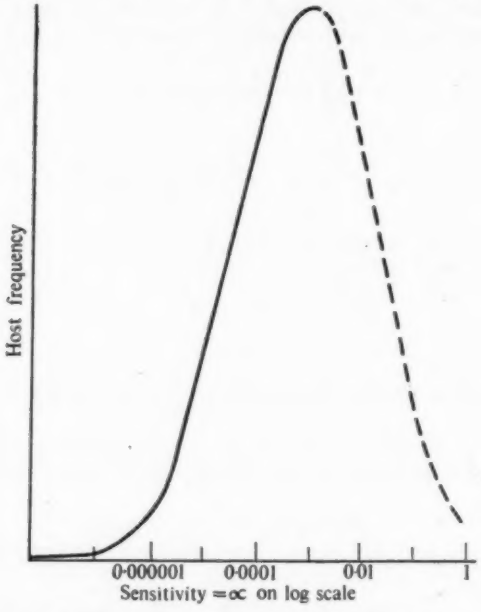

FIG. 3.—Frequency distribution of host sensitivities.
——— = estimated from the data.
– – – – – = by extrapolation.

$\log_{10}\alpha = y/\log_e 10 = \cdot 434\, y$. $E[\log_{10}\alpha] = -3\cdot 13$, $S.D.\ (\log_{10}\alpha) = 1\cdot 337$. The distribution should be truncated at $y = 0$, i.e. at $\alpha = 1$ because we defined that $\varphi(y) = 0$ for $y > 0$. As the truncation is slight it makes no appreciable difference to our estimates of the mean and variance.

Since only the high dosage levels were tested, only the upper half of the dose-response curve is established. This corresponds to the lower half of the distribution of h involving those individuals which need large doses for any reasonable chance of infection. It follows that the lower half of the distribution of h is well established by the data but the upper half (shown with dots in Fig. 3) is merely an extrapolation based on the normal fit.

If however, we consider the situation described under (f) in the theoretical introduction and we replace the formula (1) for the probability of infection by

$$1 - e^{-c\alpha}\,[1 + c\alpha + (c\alpha)^2/2! + \ldots + (c\alpha)^{g-1}/(g-1)!] \qquad (6)$$

the theory is the same except that the mean and variance of z is now

$$E[z] = -\gamma + (1 + 1/2 + \ldots + 1/(g-1))$$
$$V[z] = \pi^2/6 - (1 + 1/2^2 + 1/3^2 + \ldots + 1/(g-1)^2)$$

In this model the variance of z is even smaller and thus if it were applicable instead of (1), y would still be approximately normal.

From our results we can conclude that about 850 cells are required to produce tumours in 50 per cent of animals. This is of the same order as Hewitt (1953) found with subcutaneous inoculations of Sarcoma 37 tumour cells. Also, it is plain from our results, that between one million and two million cells are required to produce tumours in 99 per cent of animals.

Our experiments did not cover the lower end of the dose-response curve, but it is interesting to speculate that, if the distribution remained the same throughout, it suggests that one cell would produce tumours in about one to two per cent of our animals. This would not be at variance with the findings of Hauschka (1953) who succeeded in producing ascites tumours in about 15 per cent of the more susceptible suckling mice following the inoculation of single Ehrlich and Krebs ascites tumour cells. He attributed his proportion of failures only to the lack of viability of his inoculated cells but it seems probable that host resistance was, at least in part, a factor.

Another point of interest is the wide variation in sensitivities. About 850 cells are required to infect 50 per cent of mice while about two thousand times as many are necessary to infect nearly all mice. If we assume that the curve may be extrapolated towards its lower end we find that 1 to 2 per cent of mice would be infected by a single cell. It is possible to suggest that genetic incompatibility between tumour and host is responsible for the widespread variation in sensitivities. However, it is interesting to compare our results with those of Hewitt (1953). With the number of cells expressed as common logarithms (see Hewitt's Fig. 1) the standard deviation for his distribution is approximately 1·6 and ours expressed in the same units, is remarkably close to it being 1.45. Hewitt's Fig. 1 shows that the standard deviations of the distribution of sensitivities of C3H mice inoculated with C3H sarcoma is very close to that of Sarcoma 37 and our own. Hence, using the standard deviation of the distribution of sensitivities as a measure, the quoted evidence suggests that considerable variation is characteristic of transplantable mouse tumours and does not appear to be diminished by genetic homogeneity.

The similarity of Hewitt's (1953) results with Sarcoma 37 and ours with the the Ehrlich tumour is remarkable. Although the two tumours exhibit well-known differences, another similarity between them is that neither tumour is discriminating in that each appears to grow in a wide variety of white mice. Both these tumours are distinguished from the C3H sarcoma grown in the strain of origin by having a larger ED 50, but show no marked difference in variability.

When we consider the dose-response relationships of ascites tumours only (Table I and Fig. 1) we see that it is similar to that for all tumours but are shifted to the right. This suggests that, on the average, a greater number of cells are required to produce ascites than tissue growth only. In our experience well-developed Ehrlich ascites tumours always have tissue growth in addition. These findings give us no reason to think otherwise than that the production of ascites follows tissue growth if the right conditions are present, and as we have shown occurs with Sarcoma 37 ascites tumour (Warner, Kroeker and Lederman, 1957).

Fig. 2 shows the distribution of the ratios of definite ascites tumours to non-ascites tumours. One might have expected the ratio to have remained constant where less than 100 per cent of animals respond with tumour. However, this is obviously not so unless our results represent a freak of chance. We have no simple explanation for this unexpected distribution. However, we offer the following tentative and obviously speculative hypothesis.

Let us assume that the development of ascites tumours or solid tumours depends on two factors: firstly, the number of inoculated tumour cells that establish themselves in tissues and, secondly, the rate of spread of those tumour cells where rapid spreading results in the production of ascites. Also let us assume that a high degree of susceptibility in mice is associated with the ability of tumour cells to spread rapidly in such mice. Although such characteristics would be subject to a continuous distribution among the mice, the hypothesis can be illustrated in Table IV where we consider high, medium and low doses of tumour cells,

TABLE IV.—*Illustration of Hypothesis to Explain Distribution of Ratios of Ascites to Non-Ascites Tumours*

Dose	Definite ascites	Other tumours	No tumours
High	Intermediate and susceptible	Resistant	—
Medium	Susceptible	Intermediate	Resistant
Low	Susceptible	—	Resistant and intermediate

resistant, susceptible and intermediate mice which develop definite ascites tumours, other tumours, or no tumours.

The high proportion of ascites tumours at high dosages is due to the large number of inoculated cells. Where the dose is large enough practically all mice develop ascites tumours. When the dose is diminished all mice develop tumours but the resistant ones fail to develop ascites tumours. With medium sized doses the susceptible mice develop definite ascites, the intermediate other tumours and the resistant no tumour at all. Low doses fail to cause any kind of tumours in resistant and intermediate animals—those susceptible mice that are infected are so susceptible that, once infected, they will allow rapid spread of tumour and hence the development of ascites. This would account for the high proportion of ascites tumours following small doses of tumour cells. This explanation finds some support from the work of Hauschka (1953) who found that, following the inoculation of single cells into suckling mice, all tumours that developed were ascites tumours.

SUMMARY

The dose-response curve of the Ehrlich ascites tumour in mice was investigated in an attempt to learn something of the viability of tumour cells and the susceptibility of the hosts. The theoretical background of such experiments is discussed.

Six experiments were carried out in which serial dilutions of a suspension of tumour cells were inoculated intraperitoneally into mice. Observations were

obtained on a total of 614 mice of which 386 developed typical ascites tumours and 129 other types of tumour growth.

The results were subjected to probit analysis from which it was concluded that approximately 850 tumour cells would produce tumours in 50 per cent of animals and that host susceptibility in the strain of mice used was variable. Consequently, no conclusion could be drawn concerning the viability of the tumour cells. However, it was possible to determine the distribution of the log "sensitivities" of the host-cell system where "sensitivity" is the probability of infection of an animal by a single cell. It was noted that this distribution was normal with considerable variance and that its parameters were apparently in fairly close agreement with those found for Sarcoma 37 by another author. Also the variance of these tumours was of the same order as a C3H tumour grown in the strain of origin although their ED 50 was considerably greater. It was concluded that sensitivity of these transplantable mouse tumours showed considerable variation which did not appear to be diminished by genetic homogeneity.

The distribution of the proportions of mice developing ascites tumours did not appear to be markedly different from that for all types of tumours apart from a shift to the right.

The ratio of ascites tumours to those without ascites exhibited a curious distribution when plotted against doses of cells. It was high with high doses, fell with intermediate doses and then rose again with very low doses. An attempt was made to provide an explanation for this phenomenon.

It is a pleasure to acknowledge the painstaking technical assistance of Brian Moore.

REFERENCES

ARMITAGE, P. AND SPICER, C. C.—(1956) *J. Hyg., Camb.*, **54**, 401.
FINNEY, D. J.—(1952) 'Probit Analysis', Second edition, Cambridge (University Press).
HALDANE, J. B. S.—(1939) *J. Hyg., Camb.*, **39**, 289.
HAUSCHKA, T. S.—(1953) *Trans. N.Y. Acad. Sci.*, **11**, 16, 64.
HEWITT, H. B.—(1953). *Brit. J. Cancer*, **7**, 367.
HOSKINS, J. M., MEYNELL, G. G. AND SANDERS, F. K.—(1956) *Exp. Cell Res.*, **11**, 297.
IRWIN, J. O.—(1942) *J. Hyg., Camb.*, **42**, 328.
ISAACS, A.—(1957) "Particle Counts and Infectivity Titrations for Animal Viruses," in 'Advances in Virus Research.' New York (Academic Press Inc.) Vol. IV, pp. 112–155.
WARNER, P., KROEKER, H. AND LEDERMAN, J. M.—(1957) *Brit. J. Cancer*, **11**, 93.

CARCINOGENESIS IN THE PITUITARY DWARF MOUSE. THE RESPONSE TO METHYLCHOLANTHRENE INJECTED SUBCUTANEOUSLY

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MOST workers interested in the role of pituitary hormones in the induction of tumours by carcinogenic compounds or by X-rays have studied these processes in hypophysectomized animals. Bickis, Estwick and Campbell (1956) availed themselves of another experimental tool, the pituitary dwarf mouse. In this animal dwarfism is due to a genetical defect. The acidophil cells are absent from the adenohypophysis which consequently fails to secrete growth hormone in measurable amounts.

Published results indicate that the effect of hypophysectomy on carcinogenesis is not uniform. In the absence of the pituitary azo dyes and aminofluorene derivatives fail to induce liver tumours (Griffin, Rinfret and Corsiglia, 1953; O'Neal, Hoffman, Dodge and Griffin, 1958) whereas carcinogenesis by locally acting hydrocarbons (Korteweg and Thomas, 1939) and the induction of lymphoid tumours by X-rays (Nagareda and Kaplan, 1954) is not inhibited. It was therefore surprising that Bickis *et al.* (1956) obtained only one benign skin tumour in dwarf mice with doses of methylcholanthrene which induced more than 60 per cent of carcinomas in the phenotypically normal controls.

In the present paper we submit the results obtained by the subcutaneous injection of methylcholanthrene into pituitary dwarf mice.

MATERIALS AND METHODS

In 1955 Dr. R. Ortman brought mice carrying the gene responsible for pituitary dwarfism from California to the Medical School, Dunedin. The dwarfs of the imported stock had a rather short life span. Therefore one male and one female known to be heterozygous for the dwarf gene were crossed to animals of our NZY and NZC strains. Offspring of these outcrosses were used. They were two to three months old at the beginning of the experiment. Litter mates of normal phenotype and of the same sex served as controls to the dwarfs. The average weight of the latter was 6 g. and of the former 25 g.

The animals were kept in metal boxes. A perspex covered inset and cellulose wadding was provided for the dwarfs. The mice were fed ground wheat and a mash consisting of wheat germ 200 g., dried skimmed milk 1250 g., dried yeast 250 g., pollard 400 g., cod liver oil 100 ml., butter 225 g., iodized salt 100 g. and ferrous sulphate 5 g., supplemented once weekly by greens, meat meal and lightly

boiled liver. Under these conditions the dwarfs have as long a life span as normal sized mice.

Methylcholanthrene was administered as a 0·5 per cent solution in olive oil. Each mouse received one dose of 0·1 ml. by subcutaneous injection.

In order to ascertain that the typical pituitary defect was present in all dwarfs their pituitaries were studied histologically. The following staining methods were used: for acidophils Crossmon's (1937) modification of the Mallory stain and Green's (1951) method; for basophils the PAS procedure, and fuchsin-aldehyde for the demonstration of thyrotrophs. Other tissues were stained with haematoxylin-eosin or according to van Gieson; for melanin the Masson-Fontana method was used.

RESULTS

The study of the endocrine organs of our dwarfs has assured us that they conform in all essential characters to the classical picture as reviewed by Grüneberg (1952). Already the pioneer work of Snell (1930) and of Smith and MacDowell (1930, 1931) established that pituitary dwarf mice suffer not only from lack of growth hormone but that other endocrine deficiencies are also present. Most observers were impressed by the underdevelopment of the thyroid and the immaturity of the gonads, especially of the ovaries. Recently Ortman (1956) discovered that the adenohypophysis of the dwarf contains few thyrotrophs, an observation fully confirmed in our material (Fig. 1, 2).

Since we were unable to find in the literature data on the caloric intake of pituitary dwarf mice, a factor of some importance in carcinogenesis, we have checked their food consumption and found that per gram body weight the dwarfs have a greater intake than their phenotypically normal litter mates. Seven female dwarfs with an average weight of 8·2 g. and 7 sisters of normal phenotype with an average weight of 24·5 g. were placed for 2 periods of 72 hours each into metabolic cages and fed on whole wheat grain and reconstituted full cream milk. The values given in Table I are expressed as calories per 10 g. body weight/24 hours.

TABLE I.—*Food Intake of Pituitary Dwarfs and Phenotypically normal Litter Mates*

(Calories*/10 g. body weight/24 hours)

Experiment I	Wheat	Milk	Total	Experiment II	Wheat	Milk	Total
Dwarfs	9·41	0·66	10·07	Dwarfs	8·13	0·65	8·78
Normals	4·49	2·07	6·56	Normals	4·32	2·50	6·82

* 100 g. wheat = 362 calories (Sherman, 1941).
100 g. whole dried milk = 530 calories (McCance and Widdowson, 1942).

In order to test the toxicity of methylcholanthrene for dwarfs five animals were injected with 0·5 mg. of the carcinogen. There was no serious loss of weight. Within 105 days three of these mice developed sarcomas at the site of application. The two other animals were killed at 300 and 325 days when they were still in good condition. No neoplastic lesions were found at autopsy.

Table II summarizes the results obtained in 12 dwarfs and 10 controls injected with 0·5 mg. of methylcholanthrene. Tumour development occurred in both groups with about equal speed and there was no difference in incidence. Therefore

it was not considered necessary to increase the number of experiment animals. The first lesion to be noted in a dwarf and in a phenotypically normal animal originated in the epidermis. In the latter a papilloma arose at the site of injection; it progressed to an invasive carcinoma. The dwarf lived for only one week after the lesion was discovered. The short time of survival does not allow an assessment of the potentialities of the benign neoplastic changes found at autopsy in epidermis and hair follicles (Fig. 3). Only one further epithelial tumour was found, a mammary carcinoma occurring in a female control (No. 8). All other neoplasms were sarcomas. They invaded without exception the muscles of the abdominal wall and often also the panniculus carnosus. Some of the sarcomas grew with extraordinary speed. For instance in dwarf 17 the tumour was discovered 8 days before the animal was killed. At autopsy the body weight was 7·5 g. and the weight of the tumour 735 mg. Macroscopically recognizable areas of hyperpigmentation were found in two dwarfs. They were due to an increase of melanocytes in the epidermis and to an accumulation of melanophores in the subepithelial layers of the dermis. A casual inspection of sections showing sarcoma cells in close proximity with melanophores may have suggested the diagnosis melanoma. However, melanin containing cells were found only between epidermis and panniculus carnosus and never in the subcutis (Fig. 4 and 5).

TABLE II.—*Tumours Induced by Methylcholanthrene in Pituitary Dwarfs and Phenotypically Normal Litter Mates*

Dwarf	Duration (days)	Tumour	Control	Duration (days)	Tumour
19 ♂	32*	—	—	—	—
27 ♀	59	Epithelioma	16 ♀	46	Ca. of skin
8 ♀	79	Sarcoma	24 ♂	72	Sarcoma
16 ♀	86	,,	9 ♂	86	,,
9 ♂	86	,,	28 ♀	86	,,
24 ♂	86	,,	15 ♀	100	,,
25 ♂	93	,,	27 ♀	113	,,
26 ♂	100	,,	8 ♀	120	Mammary Ca.
18 ♂	108	,,	18 ♂	177	Sarcoma
28 ♀	156	,,	19 ♂	275	—
23 ♀	268	—	17 ♂	275	—
15 ♀	275	—	—	—	—

Corresponding numbers indicate litter mates; No. 18 also litter mate of No. 17; No. 24 also of No. 25 + 26.

Duration = tumour first palpated or killed to terminate experiment.

* Died.

EXPLANATION OF PLATES

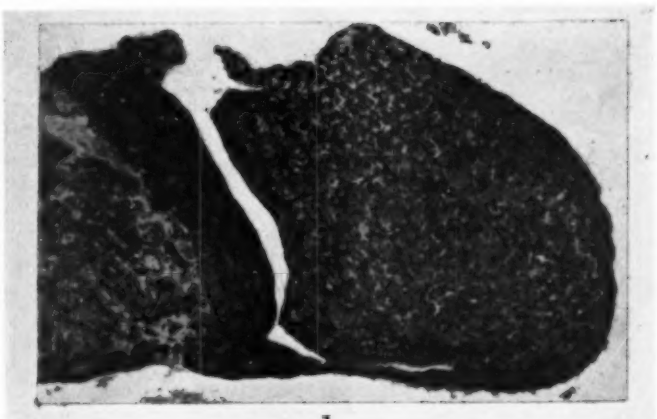
FIG. 1.—Pituitary of dwarf showing half of anterior lobe with very few small thyrotrophs (dark cells). Fuchsin-aldehye-Haematoxylin. × 120.

FIG. 2.—Central area of anterior lobe of phenotypically normal mouse. Note the numerous dark thyrotrophs. Fuchsin-aldehyde-Haematoxylin. × 120.

FIG. 3.—Benign epithelial lesion (dwarf No. 27). H. and E. × 100.

FIG. 4.—Accumulation of melanophores in dermis which is invaded by sarcoma (dwarf No. 16). Silver-Haematoxylin. × 100.

FIG. 5.—Central part of sarcoma Fig. 4. H. and E. × 100.



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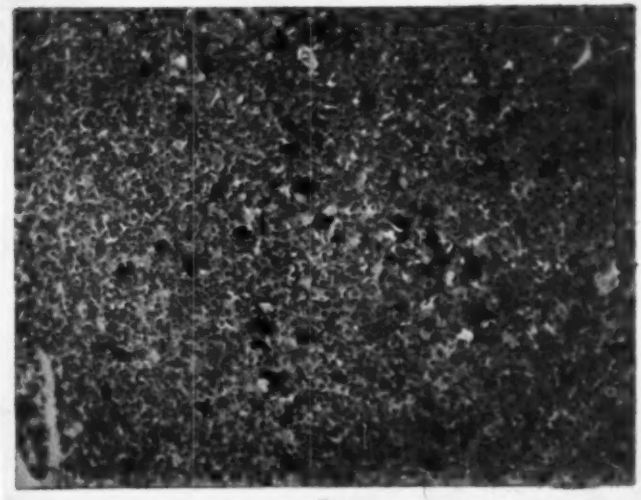

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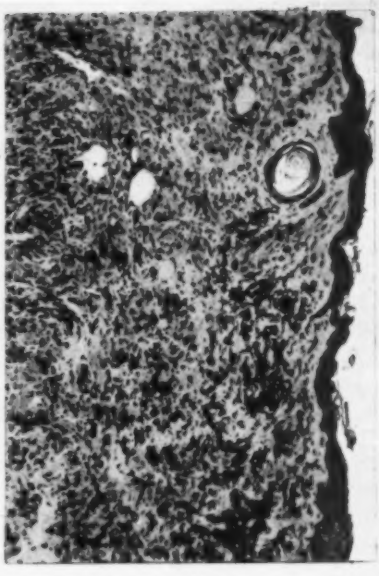

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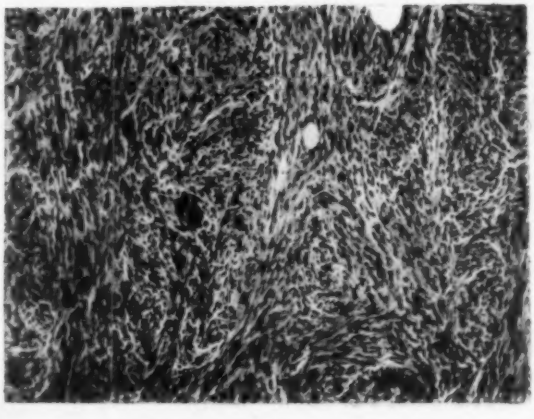

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DISCUSSION

From the results presented it is evident that the action of methylcholanthrene on connective tissue cells of the subcutis is not modified by an endocrine imbalance characterized by lack of growth hormone, thyroid deficiency and hypogonadism. Since stainable acidophils are absent from the adenohypophysis of dwarfs these animals lack probably also prolactin. A comparison of our findings with those obtained in the hypophysectomized rat shows good agreement with the work of Zamurovitch (1953) and of Agate, Antopol, Glaubach, Agate and Graff (1955) who observed that formation of sarcomas induced by benzpyrene proceeded unimpeded in the absence of the pituitary. In contrast Moon and Simpson (1955) found in two experiments a marked inhibition of the carcinogenic action of injected methylcholanthrene after ablation of the pituitary, a discrepancy which is unexplained at present. Under our experimental conditions not even a significant retardation of tumour development was apparent as was noted by Noble and Walters (1954) in hypophysectomized rats injected with dimethyl-benzanthracene.

SUMMARY

Sarcomas induced by subcutaneous injection of methylcholanthrene develop in pituitary dwarf mice in the same manner as in their phenotypically normal litter mates.

REFERENCES

Agate, F. J. Jr., Antopol, W., Glaubach, S., Agate, F. and Graff, S.—(1955) *Cancer Res.*, **15**, 6.
Bickis, I., Estwick, R. R. and Campbell, J. S.—(1956) *Cancer*, **9**, 763.
Crossmon, S.—(1937) *Anat. Rec.*, **69**, 33.
Green, J. D.—(1951) *Amer. J. Anat.*, **88**, 225.
Griffin, A. C., Rinfret, A. P. and Corsiglia, V. F.—(1953) *Cancer Res.*, **13**, 77.
Grüneberg, H.—(1952) 'The Genetics of the Mouse'. The Hague (Martinus & Nighoff).
Korteweg, R. and Thomas, F.—(1939) *Amer. J. Cancer*, **37**, 36.
McCance, R. A. and Widdowson, E. M.—(1942) *Spec. Rep. Ser. med. Res. Coun. Lond.*, **235**, 31.
Moon, H. D. and Simpson, M. E.—(1955) *Cancer Res.*, **15**, 403.
Nagareda, S. and Kaplan, H. S.—(1954) *Proc. Amer. Ass. Cancer Res.*, **1** (2), 34.
Noble, R. L. and Walters, J. H.—(1954) *Ibid.*, **1** (2), 35.
O'Neal, M. A., Hoffman, H. E., Dodge, B. G. and Griffin, A. C.—(1958) *J. nat. Cancer Inst.*, **21**, 1161.
Ortman, R.—(1956) *J. Morph.*, **99**, 417.
Sherman, H. C.—(1941) 'Chemistry of Food and Nutrition'. New York (The Macmillan Company), p. 560.
Snell, G. D.—(1930) *Anat. Rec.*, **47**, 316.
Smith, P. E. and MacDowell, E. C.—(1930) *Ibid.*, **46**, 249.—(1931) *Ibid.*, **50**, 85.
Zamurovitch, D. A.—(1953) *Oncologia*, **6**, 190.

INFLUENCE OF THE STRAIN OF OVARIAN GRAFTS ON THE INDUCTION OF BREAST AND OVARIAN TUMOURS IN F_1 $C_{57}Bl \times IF$ HYBRID MICE BY 9 : 10-DIMETHYL-1 : 2-BENZANTHRACENE

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It has been shown that fortnightly skin paintings with an oily solution of 9 : 10-dimethyl-1 : 2-benzanthracene (DMB) induce a high incidence of breast and ovarian tumours in mice of the IF strain and its F_1 hybrids with $C_{57}Bl$ (Howell, Marchant and Orr, 1954). Similar paintings of mice of the $C_{57}Bl$ strain, on the other hand, yield a much lower incidence of both types of tumour (Marchant, 1957). All these mice lack the mammary tumour agent. Susceptibility to chemical induction of tumours of the ovary may be an intrinsic genetically controlled property of the ovarian target tissue itself, or it may be determined by factors in the internal environment of the animal. If the susceptibility were a property of the ovarian tissue itself, then grafting of ovaries from susceptible and non-susceptible mouse strains to a similar environment before treatment with the carcinogen should reveal different incidences of ovarian tumours similar to those found in intact treated animals of the pure strains. The following experiment was carried out to discover whether or not this was so using F_1 hybrid hosts, which are tolerant of grafts from either pure strain.

MATERIALS AND METHOD

The mice used as hosts for the ovarian grafts were 50 adult F_1 hybrid females derived from $C_{57}Bl$ mothers and IF fathers. At ages varying from 10 to 23 weeks, both of their ovaries were dissected from the ovarian capsules and replaced with grafts of ovaries from one of 3 different sources. Seventeen mice received ovaries from young adult $C_{57}Bl$ females, 13 from IF females and 20 from F_1 $C_{57}Bl \times IF$ females.

Between 2 and 3 weeks after ovarian grafting, 6 fortnightly skin paintings of 0·5 per cent DMB in olive oil were begun. They were applied with a pipette to 16 different spots on dorsal and ventral surfaces of the body, about 1 mg. DMB (0·2 ml.) being administered at each painting.

The mice were kept in metal boxes, 5 per box, and were fed on rat cubes known as the Thompson diet. They were inspected fortnightly for tumours and were killed when breast tumours or squamous carcinomas became large, or they were otherwise in poor condition. At autopsy solid ovarian tumours or cysts were noted and removed for histological examination, as were non-tumorous ovaries. When solid tissue of any kind was found embedded in the walls of ovarian cysts, an attempt was made to assess the amount present. This was rendered fairly

simple by the transparency of the cysts. If such tissue proved on histological examination to be solid tumour, it was included in the table of ovarian tumour incidence. Cysts in which no solid ovarian tumour tissue could be found are not included in this table because cysts are frequently obtained after grafting ovaries in normal mice. Breast tumours were also noted and most of them were removed for histological examination. Several representative sections from each ovary were examined in order to detect early tumour nodules.

RESULTS

The survival of the mice in the 3 different groups was in general determined by the rate of appearance of breast tumours. It is shown in Table I, the times being from the first DMB treatment.

TABLE I.—*Survival of DMB-treated $C_{57}Bl \times IF$ mice Bearing Grafted Ovaries*

Donors of ovaries	17 $C_{57}Bl$	13 IF	20 $C_{57}Bl \times IF$
Mean survival (months)	11·7	10·5	7·6
Range in survival (months)	4·7–15·0	7·5–14·5	4·0–13·2

Ovarian tumours

Gross appearances.—In 37 of the 50 mice ovarian cysts were found. These were frequently 1 cm. or more in diameter, and they were thin-walled and filled with clear fluid which rendered them transparent. Sometimes a small patch of opaque tissue could be seen in the cyst wall. In other cases a considerable part of the cyst wall and cavity was occupied by tumour which was usually pale yellow in colour, but occasionally pink. Eight mice had bilateral cysts and in 5 of these tumour was found in the walls of one cyst. In 4 ovaries solid macroscopic tumours were found. In 7 of the 50 mice the only ovarian remains that could be detected were fragments of bright yellow tissue embedded in a mass of fat.

Incidence.—The incidence of ovarian tumours detected in the 3 groups of animals is shown in Table II.

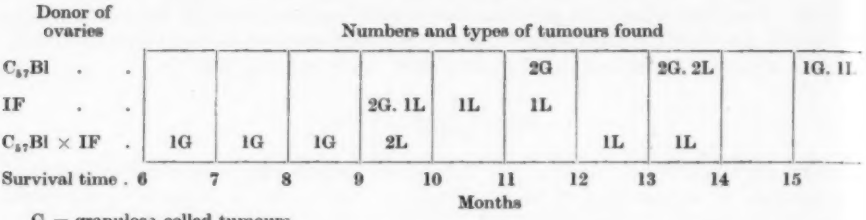
TABLE II.—*Incidence of Ovarian Tumours Induced by DMB in $C_{57}Bl \times IF$ Mice Bearing Grafted Ovaries*

Donor of ovaries		$C_{57}Bl$	IF	$C_{57}Bl \times IF$
*Tumours:	Non-atrophic	7†	2	5
	Atrophic	2	3	2
Number of mice		17	13	20
Incidence of ovarian tumours		8/17	5/13	7/20
		48%	41%	35%

* Non-atrophic indicates that the amount of ovarian tumour tissue was judged equal in size or greater than a normal ovary.
Atrophic means the amount of tumour tissue was judged to be smaller than a normal ovary.
† Two tumours were present in one mouse.

Histology.—With the exception of one spindle-celled tumour in the mouse with bilateral tumours, all ovarian tumours were granulosa-celled. Five of the other 8 tumours in $C_{57}Bl$ ovaries showed a marked degree of pseudofollicular differentiation such as was not seen in the IF or $C_{57}Bl \times IF$ ovarian grafts. The tumours found in the mice surviving longest in each group showed various

degrees of luteinisation, the earliest signs of this being found at 9 months in a $C_{57}Bl \times IF$ ovarian tumour. Fig. 1 correlates the histological type of all ovarian tumours with the survival time of the mice bearing them.



G = granulosa-celled tumours.
L = luteinising granulosa-celled tumours.

FIG. 1.—Correlation of histological type of ovarian tumour with length of survival.

Transplantability.—Two tumours, which were more than 1 cm. in diameter, were obtained in $C_{57}Bl$ ovaries. One of these, a pseudofollicular granulosa-celled tumour in a mouse surviving 13 months, was transplanted subcutaneously to a few $C_{57}Bl$ and $C_{57}Bl \times IF$ male mice. In 9 months it had grown to a similar size in some pure strain and some hybrid mice.

Breast tumours

Gross appearance.—Breast tumours appeared as subcutaneous lumps in all 3 groups of animals. They grew rapidly so that it was necessary to kill the animals bearing them within 3 to 6 weeks after their appearance. Multiple palpable tumours occurred in several mice.

Incidence.—The incidence of breast tumours which appeared in the 3 groups of animals is given in Table III.

TABLE III.—*Incidence of Palpable Breast Tumours Induced by DMB in $C_{57}Bl \times IF$ Mice Bearing Grafted Ovaries*

Donor of ovaries	$C_{57}Bl$	IF	$C_{57}Bl \times IF$
Mice with 1 breast tumour	3	7	7
" " 2 " "	2	2	6
" " 3 " "	1	0	2
" " 4 " "	0	0	1
Number of mice	17	13	20
Incidence of mice with breast tumours	6/17	9/13	16/20
	35%	69%	80%

A χ^2 test on the figures in Table III showed that the incidence of breast tumours in mice with $C_{57}Bl$ ovaries was significantly lower than in those with $C_{57}Bl/IF$ ovaries ($P < 0.01$), but the difference between those with $C_{57}Bl$ and IF ovaries was not quite significant (P about 0.065).

Histology.—The breast tumours were adenocarcinomas. In about two-thirds of those examined there was secretion and in more than half there was abundant fibroblastic stroma. Squamous metaplasia was seen in about one-third of the tumours examined and was generally quite slight in amount.

Rate of appearance.—The rate of appearance of breast tumours in the 3 groups of animals was very different. The earliest appeared in a mouse with $C_{57}Bl \times IF$ ovaries surviving 4 months, after which there was a steady increase in their numbers in this group of mice. On the other hand the earliest to appear in mice with $C_{57}Bl$ ovaries was in a mouse surviving 11 months. In mice with IF ovaries the earliest breast tumour was in a mouse surviving 9 months.

Fig. 2 correlates the number of mice bearing breast tumours with their survival.

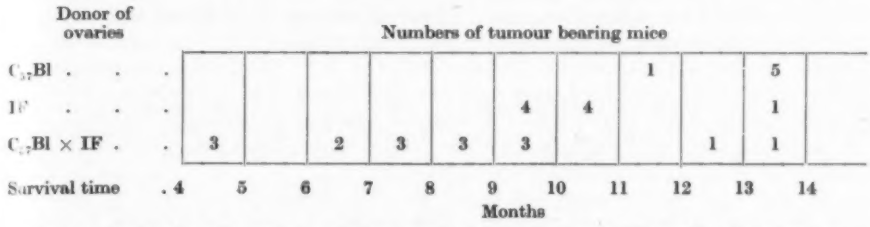


FIG. 2.—Correlation of the number of mice bearing breast tumours with length of their survival.

Other tumours

In 7 of the 17 mice with $C_{57}Bl$ ovaries, squamous carcinomas of the skin appeared earlier than the breast tumours. In the other 33 mice such a tumour was found in only 1 mouse which had $C_{57}Bl \times IF$ ovaries. One mouse developed a subcutaneous sarcoma, one a uterine sarcoma, two had tumours which probably arose in salivary glands. Several mice had enlarged spleens or a generalised lymphomatosis.

DISCUSSION

Ovarian tumours

It would appear from Table II that the incidence of ovarian tumours was very similar in the 3 groups of hybrid mice, whatever the origin of their ovaries. The slightly higher incidence in the mice with $C_{57}Bl$ ovaries was probably due to the longer survival of these animals allowing more time for ovarian tumours to arise. However, since ovarian tumours are generally rather slow in growing, it is likely that tumour nodules would already have been present and detected in most of the mice with $C_{57}Bl \times IF$ or with IF ovaries which had to be killed early because of breast tumours. It does not seem likely, therefore, that the incidence of ovarian tumours in the 3 groups was substantially affected by length of survival.

The incidence of 48 per cent of ovarian tumours induced in hybrid mice with $C_{57}Bl$ ovaries was considerably greater than the 11 per cent induced in intact $C_{57}Bl$ mice (Marchant, 1957). Since $C_{57}Bl$ ovaries were capable of giving a good yield of ovarian tumours when transplanted to $C_{57}Bl \times IF$ mice, it would seem fair to infer that the susceptibility of ovaries to tumour induction is not determined entirely by the intrinsic properties of the ovaries themselves, but is influenced by factors in the internal environment of the hosts.

Fig. 1 correlates the histological appearance of the ovarian tumours with the survival of the mice bearing them. The earliest granulosa-celled tumours found in each group of animals did not show signs of luteinisation, but the later ones

almost invariably did show it. This may simply mean that the luteinisation is a matter of maturation of the tumour cells, or it may be caused indirectly, for instance, by a change in quality of pituitary secretion after continued oestrogen secretion by the granulosa-celled tumours.

Breast tumours

It is known that breast development and tumour induction in mice are profoundly affected by ovarian secretion. From the result of the present experiment it would appear that in the same $C_{57}Bl \times IF$ hybrid hosts the strain of the ovarian grafts had considerable influence on the induction of breast tumours by DMB. Table III and Fig. 2 show a higher incidence and more rapid development in mice with $C_{57}Bl \times IF$ ovaries. Mice with $C_{57}Bl$ ovaries had the lowest incidence of breast tumours and they took longest to develop. Mice with IF ovaries were intermediate in incidence and latent period.

It might be argued that the $C_{57}Bl$ and IF ovarian grafts did not take so well in the F_1 hybrid hosts as did ovaries grafted from other hybrids and that the difference in breast tumour incidence could have been due to impaired graft function. Theoretically all the grafted ovaries should have been genetically compatible. A good yield of ovarian tumours was obtained in all three groups and in the hybrid mice with $C_{57}Bl$ ovaries the ovarian tumour incidence was actually greater than that found previously in mice of the $C_{57}Bl$ strain. There was, therefore, no reason to suspect that the grafts of pure strain ovaries failed to establish themselves well. It seemed, then, that the different incidences of breast tumours found in this experiment were more likely to result from strain differences in the ovaries themselves than in differences in graft survival.

An investigation of the ovaries from the 3 groups of donor mice used in the experiment revealed a marked difference in size. The mean weight of 24 ovaries from young adult $C_{57}Bl \times IF$ mice was found to be 9·4 mg., that of 27 IF ovaries being 7·6 mg. and that of 10 $C_{57}Bl$ ovaries being 3·1 mg. This difference in size is probably determined to some extent by genetic factors, but is also affected by the number of corpora lutea present in them. $C_{57}Bl$ ovaries contain very few corpora lutea, while IF ovaries contain very many and $C_{57}Bl \times IF$ ovaries even more. When kept 5 to a box, as in the experiment reported here, IF and $C_{57}Bl \times IF$ female mice become pseudopregnant, while $C_{57}Bl$ mice do not. In the pseudopregnant state the corpora lutea of mice become functional, secreting progesterone, whereas the normal mouse corpus luteum is said to be non-functional (Mühlbock, 1956). This is illustrated by the fact that $C_{57}Bl$ mice have a normal oestrus cycle averaging 4 to 5 days while in IF and $C_{57}Bl \times IF$ mice the progesterone secretion maintains long periods of dioestrus so that the whole oestrus cycle lasts about twice as long as in $C_{57}Bl$ mice.

The results of the present experiment show that the incidence and speed of induction of breast tumours by DMB varied directly with the mean size of the strain of ovarian grafts they were bearing, with the number of corpora lutea present in the grafts and with the level of progesterone normally secreted by ovaries of the strains concerned. It may be inferred that progesterone secretion by the grafted ovaries had a promoting effect on breast tumour induction by DMB. In this connection Jull (1954) has shown that breast tumour induction by methylcholanthrene (MC) was promoted by administration of progesterone

and later (1956) he showed that both MC and DMB had progesterone-mimetic effects on acinar development in mouse breasts.

The increased incidence of breast tumours in hybrid mice with $C_{57}Bl$ ovarian grafts over normal $C_{57}Bl$ mice treated with DMB might also be explicable in terms of the promoting effect of progesterone. It is conceivable that, when $C_{57}Bl$ ovaries were transplanted to $C_{57}Bl \times IF$ hosts, the mice were still susceptible to become pseudopregnant. If such were the case, the $C_{57}Bl$ ovaries may have increased in size after establishment of the grafts and formed functional corpora lutea secreting progesterone.

Skin tumours

The considerably higher incidence of skin tumours in mice with grafts of $C_{57}Bl$ ovaries paralleled the higher incidence of these tumours found previously in $C_{57}Bl$ mice treated with DMB in oil, as compared with similarly treated IF and $C_{57}Bl \times IF$ mice. It is possible that this may also be a reflection of the level of hormones secreted by the ovarian grafts, though the interpretation is difficult.

Oestrogen appears to be a powerful stimulator of mitosis in mouse epidermis (Bullough and van Oordt, 1950). Gilmour (1937) found the carcinogenic response of the skin to benzpyrene paintings was increased by treatment of both males and females with oestrone. Marchant (1959) has also found that after weekly skin paintings of outbred albino mice with MC in acetone, papillomas appeared earlier in intact animals than in castrates. This was particularly noticeable in females and would incidate that oestrogen has a promoting effect in skin carcinogenesis by MC. In the present experiment the grafts of $C_{57}Bl$ ovaries in the $C_{57}Bl \times IF$ hosts might have produced a higher level of oestrogen than grafts of IF or $C_{57}Bl \times IF$ ovaries, which could have been responsible for promoting the induction of skin tumours in the mice with $C_{57}Bl$ ovaries.

SUMMARY

Both ovaries were removed from F_1 $C_{57}Bl \times IF$ mice and replaced with grafts of ovaries from mice of the $C_{57}Bl$ strain (low incidence of chemically-induced ovarian and breast tumours), or from IF (high), or from similar F_1 $C_{57}Bl \times IF$ (high) mice.

All mice were then painted 6 times at fortnightly intervals with 1 mg. of 9 : 10-dimethyl-1 : 2-benzanthracene in olive oil.

The incidence of ovarian tumours which appeared in all three groups of animals was quite high. The tumours were granulosa-celled and those found in the longest survivors in each group of mice showed luteinisation. It is concluded that the susceptibility of the ovary to chemical induction of tumours is not a purely intrinsic property of the ovarian tissue itself, but is influenced by factors in the internal environment of the animals.

The highest incidence and most rapid appearance of breast tumours occurred in the group of mice with $C_{57}Bl \times IF$ ovaries and the lowest incidence and slowest appearance occurred in mice with $C_{57}Bl$ ovaries. These results are discussed in terms of promotion of breast tumour induction with DMB by progesterone.

Skin tumours appeared in a number of mice with $C_{57}Bl$ ovaries, but in only one other mouse with grafts of $C_{57}Bl \times IF$ ovaries.

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REFERENCES

BULLOUGH, W. S. AND VAN OORDT, G. J.—(1950) *Acta endocr., Copenhagen*, **4**, 291.
GILMOUR, M. D.—(1937) *J. Path. Bact.*, **45**, 179.
HOWELL, J. S., MARCHANT, J. AND ORR, J. W.—(1954) *Brit. J. Cancer*, **8**, 635.
JULL, J. W.—(1954) *J. Path. Bact.*, **68**, 547.—(1956) *Acta Un. int. Cancr.*, **12**, 653.
MARCHANT, J.—(1957) *Brit. J. Cancer*, **11**, 452.—(1959) *Ibid.*, **13**, 106.
MÜHLBOCK, O.—(1956) *Advanc. Cancer Res.*, **4**, 371.

STUDIES ON THE INCORPORATION OF AN UNNATURAL AMINO ACID, *p*-DI-(2-HYDROXY[$^{14}C_2$]ETHYL)AMINO-L-PHENYLALANINE, INTO PROTEINS

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AMINO acids bearing the nitrogen mustard group, i.e. di-(2-chlorethyl)amino-, have been found to be effective inhibitors of the Walker carcinoma in rats (Bergel and Stock, 1953, cf. Stock, 1958). Furthermore, in the case of one of these compounds, namely *p*-di-(2-chlorethyl)aminophenylalanine (Bergel and Stock, 1954) it was found (Bergel and Stock, 1953; Koller and Veronesi, 1956) that the L-isomer was more active than the D-isomer in various biological systems (Fahmy and Fahmy, 1956; Elson, 1958). This effect of the configuration of the amino acid portion of the molecule on the activity suggested that the action of the L-compound might be influenced by its possible incorporation into proteins. Cohn (1957) attempted to obtain evidence for this incorporation into protein by administering *p*-di-(2-chlorethyl)amino-DL-phenyl[β-^{14}C]alanine (Bergel, Burnop and Stock, 1955) to a rat and subsequently isolating the tissue proteins of the animal. He found that the proteins of the tumour, liver, blood and kidney were all labelled and that most of the activity was retained after the labelled protein had been treated with reagents which would be expected to break ester and ether bonds. However, he also found that liver protein and plasma albumin became firmly labelled after treatment with the labelled mustard *in vitro* under conditions in which no protein synthesis could be expected. Furthermore, similar results were obtained *in vitro* using the drug after hydrolysis, which was shown to contain a complex mixture of degradation products and not only *p*-di-(2-hydroxyethyl)-amino-DL-phenyl[β-^{14}C]alanine.

In view of the complicated and inconclusive nature of these results it was thought worthwhile to synthesize *p*-di-(2-hydroxy[$^{14}C_2$]ethyl)amino-L-phenylalanine and examine its behaviour. This compound having no reactive chloro-groups would not appear to be capable of reacting with intact proteins via these groups and therefore its presence in proteins in a firmly bound form could be taken as evidence of incorporation through peptide bond formation.

EXPERIMENTAL

[$^{14}C_2$]Ethylene oxide was prepared from barium carbonate essentially by the method of Cox and Warne (1951) except that the labelled ethylene was reacted with N-bromoacetamide to produce ethylene bromohydrin (Waltz, Fields and Gibbs, 1951) which was then converted to [$^{14}C_2$]ethylene oxide.

Reaction of [$^{14}C_2$]*Ethylene oxide with Ethyl-*p*-amino-N$^{\alpha}$-phthaloyl-*L*-phenylalaninate* (Bergel and Stock, 1954). Ethyl-*p*-amino-N$^{\alpha}$-phthaloyl-L-phenylalaninate (86·5 mg.) was dissolved in 75 per cent acetic acid (0·7 c.c.) and introduced into

a thick walled glass tube which was constricted so that when later sealed off at this point the volume within the tube was approximately 3 c.c. The tube was attached to a high vacuum line cooled in liquid nitrogen and evacuated. [$^{14}C_2$]-Ethylene oxide (23·5 mg.) previously measured and kept frozen in liquid nitrogen was released into the vacuum line and condensed into the tube containing the amine in acetic acid. The reaction tube was sealed off *in vacuo* and allowed to warm to room temperature, and then heated at 170°C for 40 hours. The tube was allowed to cool, opened and the contents transferred to a flask and concentrated to dryness, yield pale yellow gum, 130 mg. This product was refluxed with 6N hydrochloric acid (3 c.c.) for 5 hours. On cooling phthalic acid separated as a crystalline solid, and was filtered and washed with 6N acid. The filtrate and washings were concentrated to dryness, water added and again concentrated. This last step was repeated twice more to remove all traces of hydrochloric acid, before dissolving in methanol and concentrating to dryness. The resulting product was a deliquescent brown solid, which was examined by paper chromatography (descending) using Whatman No. 4 paper and the solvent, *n*-butanol : propanol : 0·1 N ammonia in the proportions of 2 : 1 : 1, clarified if necessary with propanol. The substances were located on the paper by dipping in a 0·4 per cent solution of ninhydrin in chloroform containing 2·0 per cent acetic acid.

The reaction product was found to contain three ninhydrin positive compounds of Rf 0·14 (brown colour), 0·23 (purple) and 0·29 (purple). The slowest running substance giving the atypical ninhydrin colour was shown to be *p*-amino-L-phenylalanine by comparison of its behaviour on paper chromotography with that of an authentic specimen. Similarly the compound of Rf 0·29 was shown to be *p*-di-(2-hydroxyethyl)amino-L-phenylalanine. An authentic sample of *p*-mono-(2-hydroxyethyl)-amino-L-phenylalanine was not available but the general behaviour of the compound of Rf 0·23 suggested that it was this compound.

A cellulose column (55 × 3·5 cm.) was prepared using Whatman standard grade cellulose powder and the solvent mixture used for paper chromatography.

The solid obtained from the reaction described above was dissolved in 5 c.c. of the same solvent and applied to the column. When this had been absorbed on the column, development was continued with the same solvent mixture ; 25 c.c. fractions were collected automatically and were examined by drying down 0·1 c.c. samples on 1 sq. cm. polythene planchet. The counting was carried out using a mica end-window Geiger-Muller counter connected to a scaling unit. Under these conditions there was zero self-absorption by the samples. After counting, the content of the planchets was dissolved in a drop of water, applied to paper and chromatographed as described above.

It was found that fractions 17 to 20 contained a radioactive product which was ninhydrin negative. Fractions 24–27 contained *p*-di-(2-hydroxy[$^{14}C_2$]ethyl)-amino-L-phenylalanine and fractions 29–32 contained *p*-mono-(2-hydroxy[$^{14}C_2$]-ethyl)amino-L-phenylalanine. Fraction 28 contained some of both compounds so was not used. The *p*-amino-L-phenylalanine was obtained in fractions 33–35. The appropriate fractions were bulked and concentrated.

Fractions 24–27 contained only *p*-di-(2-hydroxy[$^{14}C_2$]ethyl)amino-L-phenylalanine (16 mg.) at a calculated specific activity of 5·4 μc./mg.

Fractions 29–32 contained *p*-mono-(2-hydroxy[$^{14}C_2$]ethyl)amino-L-phenylalanine (20·5 mg.) at a calculated specific activity of 3·2 μc./mg. Both these compounds were obtained as brown solids which could not be crystallized.

In vitro experiments

For the *in vitro* and *in vivo* experiments the *p*-di-(2-hydroxy[$^{14}C_2$]ethyl)amino-L-phenylalanine was used as an aqueous solution at a concentration of 4 mg./c.c., i.e. 22 μc./c.c.

A. To a solution of serum albumin (100 mg.) in water (5 c.c.) was added 0·1 μc. of *p*-di-(2-hydroxy[$^{14}C_2$]ethyl)amino-L-phenylalanine and the mixture incubated at 37° for 24 hours. The solution was dialysed at 0° overnight and then freeze-dried. The radioactivity of the resulting protein, as an infinitely thick sample, was counted in the usual way and was found to be insignificant.

B. A rat liver (9 g.) was homogenized in 0·9 per cent sodium chloride solution (45 c.c.). To 10 c.c. of this homogenate was added 0·2 μc. of *p*-di-(2-hydroxy-[$^{14}C_2$]ethyl)amino-L-phenylalanine and the mixture incubated at 37° overnight. The protein was then isolated in the usual way (Cohn, 1957) and its radioactivity counted. This activity was found to be insignificant.

In vivo experiments

One Wistar male rat (230 g.) previously starved for 24 hours was injected intraperitoneally with 13 μc. (i.e. 30 μc./kg.) of *p*-di-(2-hydroxy[$^{14}C_2$]ethyl), amino-L-phenylalanine in aqueous solution (0·6 c.c.). After 75 minutes the animal was killed (neck broken) and the following tissues removed; (*a*) Kidney, (*b*) Liver, (*c*) Spleen, (*d*) Thymus, (*e*) Blood.

A sample of each of these tissues was treated for the isolation of total protein as described by Cohn (1957) and the final dry solids were plated on 1 sq. cm. polythene planchets and the radioactivity counted at infinite thickness. In a further similar experiment, 10 μc. of the labelled amino acid analogue were injected into a rat and the tissues of kidney, liver and spleen isolated as before. The result of these experiments is given in Table I.

TABLE I.—*Radioactivity Found in the Protein of Several Tissues of the Rat after the Administration of* p-*di-(2-hydroxy*[$^{14}C_2$]*ethyl)amino-*L*-phenylalanine*

Tissue examined	Counts/min. Infinitely thick sample on 1 sq. cm. disc (1 μc./g. = 580 c./m.)	
	Experiment 1	Experiment 2
Kidney protein	9	12
Liver protein	3	*Nil*
Blood protein	2	—
Spleen protein	2	2
Thymus protein	*Nil*	—

In order to determine what radioactivity might be expected in tissue protein if the phenylalanine analogue had been incorporated, 10 μc. of L-[G-^{14}C]phenylalanine was injected into a rat and the tissue proteins isolated as in the previous experiments. The result obtained is shown in Table II.

DISCUSSION

In the preparation of di-(2-hydroxyethyl)amino compounds by the reaction of ethylene oxide and an amine, a large excess of ethylene oxide is normally

TABLE II.—*Radioactivity Found in the Protein of Several Tissues of the Rat after the Administration of* L-[*G*-^{14}*C*]*phenylalanine*

Tissue examined	Counts/min. Infinitely thick sample on 1 sq. cm. disc (1 μc./g. = 580 c./m.)
Kidney protein	385
Liver protein	485
Spleen protein	604

used to avoid leaving unchanged amine and mono-(2-hydroxyethyl)amino compounds in the reaction mixture. However, when labelled ethylene oxide is being used it is obviously uneconomical to use an excess, and so it is to be expected that a mixture of products will be obtained. Various reaction conditions were investigated in an attempt to obtain a good yield of the desired *p*-di-(2-hydroxyethyl)amino-L-phenylalanine, and the method as described in the experimental section was found most satisfactory.

The *in vitro* experiments showed clearly that the labelled phenylalanine analogue was not bound to protein under conditions in which Cohn (1957) had found resulted in firm binding of both *p*-di-(2-chlorethyl)amino-DL-phenyl[β-^{14}C]alanine and of the hydrolysis product of this compound.

The *in vivo* experiments showed that of the tissue proteins examined only the protein of kidney showed a level of activity significantly above background and even in this case the count was extremely low. These results show that *p*-di-(2-hydroxy[$^{14}C_2$]ethyl)amino-L-phenylalanine is not incorporated into proteins. It may therefore be reasonably concluded that the activity which Cohn (1957) found in tissue proteins, after the injection of *p*-di-(2-chlorethyl)amino-DL-phenyl-[β-^{14}C]alanine is not due to incorporation of this compound into protein through peptide bonds but rather to an alkylation reaction involving the "nitrogen mustard" portion of the molecule. The reason for the greater biological activity of the nitrogen mustard derivative of L-phenylalanine cannot therefore be ascribed to such an incorporation. It might be due to a more facile transport of the L-enantiomorph in comparison with the D-isomer.

SUMMARY

1. The synthesis of a hydrolysis product of an L-phenylalanine nitrogen mustard, namely *p*-di-(2-hydroxy[$^{14}C_2$]ethyl) amino-L-phenylalanine, from [$^{14}C_2$]ethylene oxide is described.

2. This compound was found not to react *in vitro* with serum albumin or the proteins of rat liver.

3. Injection of the compound into rats did not result in any significant labelling of the tissue proteins.

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REFERENCES

BERGEL, F. AND STOCK, J. A.—(1953) *Rep. Brit. Emp. Cancer Campgn.*, **31**, 6.—(1954) *J. Chem. Soc.*, 2409.
Idem, BURNOP, V. C. E. AND STOCK, J. A.—(1955) *Ibid.*, 1223.
COHN, P.—(1957) *Brit. J. Cancer*, **11**, 258.
COX, J. D. AND WARNE, R. J.—(1951) *J. Chem. Soc.*, 1893.
ELSON, L.—(1958) *Ann. N.Y. Acad. Sci.*, **68**, 828.
FAHMY, O. G. AND FAHMY, M. J.—(1956) *J. Genet.*, **54**, 146.
KOLLER, P. C. AND VERONESI, U.—(1956) *Brit. J. Cancer*, **10**, 703.
STOCK, J. A.—(1958) Ciba Foundation Symposium on ' Amino Acids and Peptides with Antimetabolic Activity '. London (J. and A. Churchill, Ltd.), p. 89.
WALTZ, D. E., FIELDS, M. AND GIBBS, J. A.—(1951) *J. Amer. chem. Soc.*, **73**, 2968.

DIFFERENCES IN THE DEOXYRIBONUCLEIC ACID CONTENT OF NUCLEI FROM SCHWANN CELLS, ENDONEURIAL CELLS AND RELATED TUMOUR CELLS

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THE fundamental importance of deoxyribonucleic acid (DNA) in the process of cell growth and reproduction has long been recognised (Davidson, 1957). Neoplastic tissue is known to be rich in nucleic acids and there is evidence that a higher than normal DNA level is found in the nucleus of the malignant cell (Stowell, 1947).

A direct comparison between tumour cells and their normal homologues is made difficult by the doubts which arise in identifying the cell types from which the tumours arise. In a few cases, such as the experimental tumours of the liver studied in detail by Thomson, Heagy, Hutchison and Davidson (1953) where the normal homologue can be identified with some degree of assurance, a higher level of DNA has been observed in the nuclei of the tumour cells.

Experimentally produced tumours associated with peripheral nerve in mice present another material in which it is possible to compare with some degree of certainty tumour cells with their normal homologues. This paper is concerned with an examination of these tumours and a comparison of the amount of DNA found in the nuclei of their cells with the nuclei of Schwann cells, both normal and proliferating.

MATERIALS AND METHOD

The estimation of the amount of DNA present in the cell nucleus by making use of the Feulgen nuclear reaction is well established (Di Stefano, 1948 ; Davidson, 1957 ; Swift and Rasch, 1956). The apparatus used for determinations of this kind is known as a Microdensitometer or Microphotometer and consists of a light source, a microscope and a photometer. A Hilger High Power Microphotometer was employed throughout this work and a magnification of 200 diameters was employed.

The method of use of this instrument has already been described (Causey and Stratmann, 1959). In brief, a magnified image of the section is projected on to the photometer, the area to be measured being delimited by a diaphragm aperture of suitable size.

The procedure of measuring the absorption of a feature such as a nucleus is as follows : First a clear area adjacent to the feature is selected and a background reading is taken (I_0). The nucleus is moved into the field covered by the aperture and a further reading is taken (I_s). T, the transmission of the feature is then given by I_s/I_0.

The Extinction, E, is defined as $\log_{10} 1/T$, i.e. $\log_{10} I_0/I_s$.

The Feulgen stain obeys the Lambert-Beer Laws (Swift and Rasch, 1956) and within the limits of concentration found in nuclei it can be said that the observed extinction is proportional to the amount of absorbing substance present in the area measured, and hence proportional to the amount of DNA present in the nuclei.

The size of the diaphragm apertures used were selected so that the absorption of a central " plug " of the nucleus was measured. The size of the aperture being such that no more than about two-thirds of the area of the average nucleus was measured. The fraction of nuclear volume measured is, however, considerably smaller than this. For example, in the case of a nucleus 10 μ in diameter, the fraction of the nuclear volume measured is of the order of 1/25 of the total volume.

All results are calculated in terms of M, the amount of absorbing substance. This is defined as $E \times A$, where $E =$ the extinction and A the area (μ^2) of the slide actually measured. For convenience the resultant figure has been multiplied by 100. It is assumed that any lack of uniformity of the " plugs " will tend to cancel out when large numbers of nuclei are measured.

The staining technique employed was substantially the same as the modified Feulgen Technique described by Rafalko (1946) except that all stages of the technique were carried out in a Histokinette Tissue Processor. Full details are given elsewhere (Causey and Stratmann, 1959). No counter-staining was carried out.

The production of the tumours associated with nerve by injection of 3 doses of the carcinogen 9, 10-dimethyl-1, 2-benzanthracene (DMBA) has been described by Causey (1959). These tumours have been classified as intra- or extra-neural on the basis of whether the perineurium is or is not disrupted. A typical intra-neural tumour is shown in Fig. 5. On removal of the tumours, a slice was placed in Zenker-Formol fixative, and fixed for 5–6 hours. The material was then washed overnight in running water and embedded in paraffin in the usual way. Later, 8 μ sections were prepared and stained as above. Fibrosarcomas were produced by injections of the same doses of DMBA into the dermis of the back.

White laboratory mice of C+ strain were used throughout. In normal animals the sciatic nerve in the thigh was removed from both legs immediately after death, cleaned of any adherent fatty and connective tissue. The nerves were fixed in Zenker-Formol fixative for 5–6 hours. As before the material was washed in running water overnight and the usual paraffin embedding procedure carried out.

During the early stages of the degeneration of a peripheral nerve, there is a marked proliferation of Schwann and endoneurial cells (Abercrombie and Johnson, 1946). Since it was desired to examine the nucleic acid content of these proliferating cells, some material was prepared in which the nerve had been sectioned 14 days prior to removal and fixation. The sciatic nerve of the mice used in these experiments was sectioned in the thigh under aseptic conditions (Causey and Stratmann, 1953). Ether was used as anaesthetic. Fourteen days after section of the nerve the mice were killed and the sciatic nerves on both sides removed as previously described. In order to obtain a sufficient number of nuclei per section for purposes of measurement, longitudinal sections of peripheral nerve, both normal and degenerating, were cut. Throughout this work section thickness was 8 μ in the case of both nerve and tumours. Typical longitudinal sections of Feulgen stained sciatic nerve are shown in Fig. 1 and 2. Fig. 1 shows normal

nerve, and Fig. 2 after 14 days degeneration. The photographs were taken with the interference microscope, to show the typical appearance of the tissue.

RESULTS

In order to establish some basis for comparison of levels of absorbing substance in the nuclei of tumours associated with peripheral nerve and the nuclei of cells of the homologous tissue, Schwann nuclei of the normal and degenerating nerve of mice were examined. The results of these measurements are shown in Table I, and in Fig. 1 and 2 typical sections of normal and degenerating peripheral nerve are shown.

TABLE I.—*Absorption Measurements of Schwann Cell Nuclei from the Sciatic Nerve of the Mouse*

Results are expressed in terms of M, the amount of absorbing substance in the area measured. Thus $M = E \times A \times 100$ where E = the extinction, A = the area measured = $2{\cdot}2\mu^2$.

Mean values for M are given together with the standard error of the mean. Number of nuclei measured is given in parenthesis after each result.

Normal nerve

$M = 24{\cdot}8 \pm 0{\cdot}8$ (104)

14 days after section of the nerve in thigh

$M = 30{\cdot}2 \pm 0{\cdot}9$ (70)

Examination of the results shown in Table I reveals that there is a slightly increased amount of absorbing substance, that is DNA, in the Schwann nuclei of the proliferating cells.

Table II shows the results obtained from an examination of the amount of absorbing substance in tumours associated with nerve. Results are given for nuclei of the two major cell types observed in the tumours. These have been classified arbitrarily into " small " and " large " nuclei. These two types compare with the tumour cell types " A " and " B " described by Caspersson and Santesson (1942). The " small " nuclei corresponding to the type " A" nuclei of Caspersson and Santesson. These are some 5–7 μ in diameter and have a relatively high concentration of DNA. The " large " nuclei, corresponding with the type " B " nuclei of Caspersson and Santesson are larger and somewhat more diffuse. The diameters of these are of the order of 10–15 μ. In Fig. 3 and 4 these two types of nuclei can be seen. The two types described can be regarded as the

EXPLANATION OF PLATE.

FIG. 1.—Feulgen stained 8μ longitudinal section of normal sciatic nerve of the mouse showing Schwann cell nuclei (magnification 450 diameters).

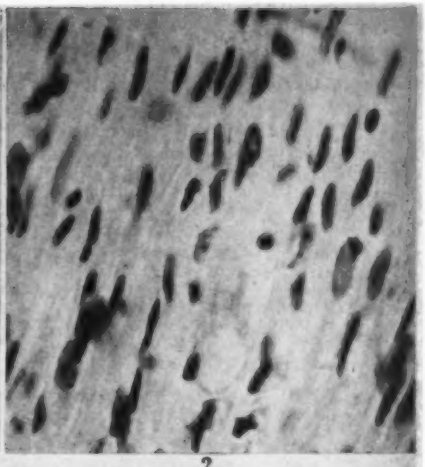
FIG. 2.—Feulgen stained 8μ longitudinal section of the sciatic nerve of the mouse 14 days after section of nerve in thigh (magnification 450 diameters).

FIG. 3.—Feulgen stained 8μ section of intraneural tumour showing " large " (L) and " small " (S) nuclei (magnification 450 diameters).

FIG. 4.—Feulgen stained 8μ section of extraneural tumour showing " large " (L) and " small " (S) nuclei (magnification 450 diameters).

FIG. 5.—Intraneural tumour in the mouse.

1



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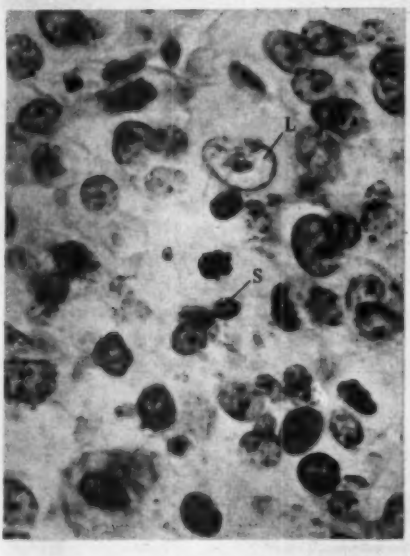

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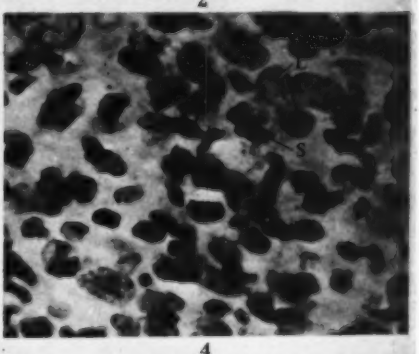

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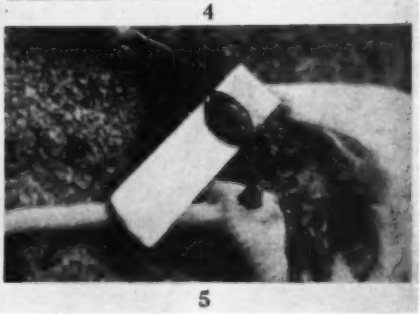

5

extreme cases and various stages of " transition " between " small " and " large " can be seen. For a further discussion see Caspersson and Santesson (1942).

Results for the nuclei of fibrosarcomas are also shown in Table II.

TABLE II.—*Absorption Measurements of Cell Nuclei from Tumours Associated with Peripheral nerve and Tumours of Fibrous Tissue in the Mouse*

Results are expressed in terms of M, the amount of absorbing substance in the area measured. Thus $M = E \times A \times 100$ where E = the extinction, A = the area measured = $2 \cdot 2\mu^2$.

Mean values for M are given together with the standard error of the mean. Number of nuclei measured is given in parenthesis after each result.

(1) " Small " nuclei in tumours " inside " nerve :

$M = 44 \cdot 2 \pm 0 \cdot 9$ (261)

(5 tumours)

(2) " Large " nuclei in tumours " inside " nerve :

$M = 31 \cdot 0 \pm 0 \cdot 7$ (260)

(3) " Small " nuclei in tumours " outside " nerve :

$M = 43 \cdot 6 \pm 0 \cdot 8$ (254)

(5 tumours)

(4) "Large " nuclei in tumours " outside " nerve :

$M = 25 \cdot 3 \pm 0 \cdot 6$ (258)

(5) " Small " nuclei in fibrosarcomas :

$M = 45 \cdot 4 \pm 0 \cdot 6$ (101)

(2 tumours)

(6) " Large " nuclei in fibrosarcomas :

$M = 26 \cdot 5 \pm 0 \cdot 8$ (102)

Note : The classification of nuclei into " Large " and " Small " follows that of Caspersson and Santesson (1942). The classification of the tumours themselves into " inside " and " outside " nerve is based on the arbitrary classification described by Causey (1959).

Examination of Tables I and II and a comparison of the results obtained shows that there is a significantly higher amount of DNA present in the nuclei of the tumour cells as compared with either normal or proliferating Schwann cells.

As far as the nuclei of the tumours themselves were concerned, examination of Table II shows that there was no detectable difference in the DNA content of nuclei from tumours " inside ", " outside " nerve or nuclei of the fibrosarcomas.

DISCUSSION

Rapidly metabolising cells are known to be richer in DNA than more quiescent cells (Davidson, 1957), and in those cases where it has been possible to compare the DNA content of malignant neoplasms with their non-malignant homologues a somewhat higher level of DNA has been shown (Stowell, 1947 ; Thomson *et al.*, 1953).

It is considered probable that the cells of those tumours associated with peripheral nerve which disrupt the perineurium have been derived from Schwann cells. Those tumours that disrupt the perineurium have been shown to occur

with significantly greater frequency when the injection of the carcinogen is made after crushing the nerve than when it is made into normal nerve, and also that in those tumours deliberately produced outside the perineurium, invasion of the perineurium is a late phenomenon. A macroscopic picture of an early intraneural tumour is shown in Fig. 5. The typical swelling of the nerve trunk in a fusiform manner is seen. It is possible that there are also cytological differences of which the most distinct is the tendency of the cells of the intraneural tumours to form alveolar clusters in contradistinction to the elongated cells of the fibrosarcomas and extraneural tumours. A comparison of the amounts of DNA in both " small " and " large " nuclei of these tumours with amounts of DNA found in both normal and proliferating Schwann cell nuclei shows that there is a somewhat greater amount of DNA in the tumour nuclei of both types, even allowing for the considerably greater diameter of the " large " nuclei.

Although there are, of course, significant differences in the DNA content of the " small " and " large " nuclei, even after allowing for differences in size of these nuclei, no difference could be observed in DNA content of nuclei in tumours classified as arising either " inside " or " outside" nerve. Neither could any difference in DNA content be shown between these nuclei and the nuclei of the fibrosarcomas of the back.

If it can be assumed that the fibrosarcomas and those tumours associated with nerve that are considered to arise " inside " nerve are in fact derived from different cell types, then it can be said that as far as the DNA content of the nuclei of the resultant tumour is concerned, there is no significant difference between tumours of different origin.

It is fairly clear, however, that an increase in DNA of the nucleus of tumour cells as compared with normal cells does occur. It is, of course, known that increase in activity of cells is reflected by an increase in DNA content of the nuclei and in the case of Schwann cells results of this kind have been reported here.

The observed differences in DNA content in tumour cells and their normal homologues may be explained by a difference in the metabolic activity of the cells. In the absence of evidence to the contrary this would appear to be the most reasonable explanation of the observations reported in this paper.

SUMMARY

1. By the use of microphotometric methods of analysis, the deoxyribonucleic acid (DNA) content of the nuclei of Schwann cells, both normal and proliferating, has been compared with nuclei obtained from the cells of tumours associated with nerve and fibrosarcomas.

2. A significantly greater amount of DNA has been found in the nuclei of both types of tumour cells as compared with both normal and proliferating Schwann cells.

3. The significance of these results is discussed.

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REFERENCES

ABERCROMBIE, M. AND JOHNSON, M. L.—(1946) *J. Anat., Lond.*, **80**, 37.
CASPERSSON, T. AND SANTESSON, L.—(1942) *Acta radiol., Stockh.*, Suppl. 46.
CAUSEY, G.—(1959) *Acta Un. int. Cancr.*, **15**, 142
Idem AND STRATMANN, C. J.—(1953) *J. Physiol.*, **121**, 215.—(1959) *J. Anat., Lond.* (in press).
DAVIDSON, J. N.—(1957) 'The Biochemistry of the Nucleic Acids'. 3rd edition. London (Methuen).
DI STEFANO, H. S.—(1948) *Proc. nat. Acad. Sci., Wash.*, **34**, 75.
RAFALKO, J. S.—(1946) *Stain Tech.*, **21**, 91.
STOWELL, R. E.—(1947) *Symp. Soc. exp. Biol.*, No. 1, p. 190.
SWIFT, H. AND RASCH, ELLEN.—(1956) 'Microphotometry with Visible Light'. In 'Physical Techniques in Biological Research'. Ed. Oster, G. and Pollister, A. W. Vol. III, pp. 353–400. New York (Academic Press Inc.).
THOMSON, R. Y., HEAGY, F. C., HUTCHISON, W. C. AND DAVIDSON, J. N.—(1953) *Biochem. J.*, **53**, 460.

THE INFLUENCE OF DIETARY PROTEIN ON THE METABOLISM OF RIBONUCLEIC ACID IN RAT HEPATOMA

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In a previous study (Clark, Naismith and Munro, 1957) we examined the uptake of [^{14}C]glycine and of ^{32}P by the liver ribonucleic acid (RNA) of normal rats and found conditions under which the incorporation of these precursors was extensively influenced by the protein content of the diet. In particular, rats fasting after a meal containing protein had a much lower uptake of these labelled precursors than animals fasting after a protein-free diet; uptake was rapidly restored by feeding protein. A considerable degree of sensitivity of liver RNA metabolism to the immediate supply of amino acids was thus established.

It seemed of interest to know whether a tumour derived from liver cells would still exhibit the same fluctuations in RNA metabolism in response to changes in the dietary supply of protein or whether its metabolic activity would be less dependent on dietary conditions. Experiments have accordingly been carried out on rats bearing a transmissible hepatoma implanted subcutaneously, and the responses of the RNA in the tumour and in the normal liver to dietary protein intake have been compared. Whereas the livers of the tumour-bearing animals showed the usual low isotope uptake when the rats were fasted after a meal of protein, the tumour cells did not do so. The hepatoma cells are thus less dependent on dietary conditions than are the parent liver cells.

A preliminary account of these experiments has already appeared (Munro and Clark, 1958).

EXPERIMENTAL METHODS

1. *General techniques*

In the experiments described below, the response of RNA metabolism in liver tumour cells to changes in dietary protein intake has been compared with the response of RNA in normal liver and in regenerating liver.

Diets and isotope administration.—Rats of about 200 g. body weight were used in the experiments. For about a week before injection of the isotopes, the animals were fed on synthetic diets (Munro and Naismith, 1953), either containing protein or free from protein. These diets provided about 1200 cal./sq.m. body surface area/day and were given in two portions. The vitamins, minerals and a part of the dietary carbohydrate and fat were fed at 9 a.m. and the rest of the diet (including any protein in the diet) was given at 5 p.m. The animals soon learned to consume these meals rapidly, so that on the morning after the last day of feeding they were all in the post-absorptive state. Rats which had received the protein-free diet and some which had eaten the diet containing protein were then injected intramuscularly at 9 a.m. with ^{32}P (20 μc./100 g. body weight) and

with [2-^{14}C]glycine (10 μc./100 g. body weight in the experiments on tumour-bearing animals and 5 μc./100 g. body weight in the studies on liver regeneration). These animals remained fasting until killed 3 or 6 hours later. In addition, some of the rats which had been on the protein-containing diet were fed 2·5 g. of casein solubilized with 0·15 g. $NaHCO_3$, one hour before isotope injection. Thus RNA metabolism could be compared in three groups of animals, (a) those fasting after the protein-free diet, (b) those fasting after the protein-containing diet, and (c) those actively absorbing amino acids from the gut.

Removal and analysis of tissues.—Rats from each of the three dietary groups were killed at 3 and at 6 hours after isotope injection. The tissue (liver or tumour) was excised and homogenized in a Nelco blendor in 5 volumes of ice-cold water for 3 minutes and 0·5 volumes of 30 per cent trichloroacetic acid was added. The homogenate was then submitted to procedures described previously (Clark *et al.*, 1957) for quantitative determination of protein N, RNA phosphorus (RNAP) and deoxyribonucleic acid phosphorus (DNAP), and for assessing the specific activity of ^{32}P in the tissue inorganic phosphate and RNA phosphorus. The specific activities of [^{14}C]glycine in the free amino acid pool (trichloroacetic acid-soluble free glycine) and in the glycine portion of the RNA purine bases of the tissue were measured on samples of dinitrophenylglycine prepared from these sources by methods described previously (Clark *et al.*, 1957). These samples were applied evenly to nickel planchets and assayed for radioactivity using an end-window Geiger-Müller counter. The count was prolonged sufficiently to bring the standard error of the count below 5 per cent. A correction for self-absorption by the sample was employed.

In the experiments on liver regeneration, uptake of ^{32}P by deoxyribonucleic acid (DNA) was also measured in order to be certain that the liver was in a state of active cell multiplication at the time of killing. The procedure used for preparing DNA for assay of its radioactivity was similar to that recommended by Daoust and Hooper (1957). The DNA was separated from the RNA by the Schmidt-Thannhauser procedure as in the case of its quantitative determination, except that the DNA-containing precipitate obtained on acidifying the alkaline digest of nucleic acids was washed three times with 10 per cent trichloroacetic acid before the specific activity of ^{32}P in the DNA was measured. The low uptake of ^{32}P by the liver DNA of the control (laparotomized) rats suggests that radioactive contaminants were adequately removed from the DNA by this procedure.

Method of expressing radioactive uptake.—The uptakes of ^{32}P and of [^{14}C]-glycine by the various tissue components were first calculated in the form of specific activities (counts/min./100 μg. P or glycine). The specific activities of [^{14}C]glycine samples isolated from the purine bases were then expressed as a percentage of the specific activity of the glycine in the free amino acid pool of the tissue; similarly, the specific activities of ^{32}P in the RNA and the DNA were related to that of the tissue inorganic phosphate. These provide relative specific activities which allow for differences in the activities of the precursor pools of different animals. In some experiments, a measure of the total incorporation/cell was also calculated by multiplying the relative specific activity of RNA by the amount of RNAP/mg. DNAP; this is referred to as the total relative activity.

Deoxyribonucleic acid content of liver and tumour cell nuclei.—Nuclei were prepared by the citric acid procedure, as modified by Smellie, Humphrey, Kay and Davidson (1955). In order to obtain clean nuclei, the nuclear fraction was washed

with 0·01 M citric acid in the manner described by these authors, the efficacy of washing being controlled microscopically. The nuclei, suspended in 0·01 M citric acid, were counted in a haemacytometer; a portion of the suspension was also taken for DNA phosphorus determination (Clark *et al.*, 1957). The DNAP content per nucleus was then expressed in pg. (picograms, that is, 10^{-12} g.).

II. Experiments with the transmissible hepatoma

The main experiments described in this paper were carried out with rats bearing a transmissible hepatoma implanted subcutaneously. The effect of diet on RNA metabolism in the tumour and in the normal liver of the tumour-bearing animals were compared.

Rats of the August strain were inbred to carry the transmissible hepatoma. Dr. K. S. Kirby of the Chester Beatty Institute very kindly supplied us with the transmissible tumour. This originated from an intrahepatic hepatoma produced by feeding 4-fluoro-dimethylaminoazobenzene to rats, and was maintained by subcutaneous inoculation of a suspension of tumour cells. The developed tumour was a solid, fleshy mass. Histologically, the tumour conformed to a Type II hepatoma (Edwards and White, 1941). There were some areas of necrosis in all tumours, but round-cell infiltration and fibrous tissue development were slight.

About 3 weeks before each series of isotope experiments, male rats from the colony were inoculated in the right flank with 1 ml. of a suspension of tumour cells. From this group, animals showing the same degree of tumour development were selected and, for a period of 7 days before injection of the isotopes, some were fed with the protein-containing diet and others with the protein-free diet described above. At the end of this period, the rats were injected with ^{32}P and with [2-^{14}C]glycine, some animals being in the post-absorptive state, others having been fed casein just prior to injection. The livers and tumours from rats in each of these dietary groups were analyzed as described above.

III. Experiments with intrahepatic tumours

In order to be certain that the differences observed between the metabolism of RNA in the transmissible hepatoma and in the normal liver could not be due to the subcutaneous position of the tumour, an experiment was carried out in which tumours were produced in the liver by feeding dimethylaminoazobenzene. Male rats of about 150–180 g. body weight were fed on a carcinogenic diet (Miller, Kline and Rusch, 1946) containing 0·03 per cent dimethylaminoazobenzene for the first month and 0·06 per cent of this carcinogen thereafter. After 6 months liver tumours became apparent and the surviving animals were then fed on the stock diet for a further 2 weeks before commencing the final experimental period.

In this experimental period, some of the tumour-bearing rats were fed for 6 days on the protein-containing diet and others on the protein-free diet. On the morning of the 7th day, they were injected in the fasting state with ^{32}P and [2-^{14}C]glycine and were killed 6 hours later. In this experiment, none of the animals was fed casein at the time of isotope injection. The tumours were dissected free from the rest of the liver, representative portions of the tumour and of the non-cancerous parts of the liver being taken for measurement of isotope uptake. The tumours and non-cancerous portions of the liver were also examined histologically. The tumours showed some areas of degeneration and neutrophil

infiltration ; the areas of liver not transformed into tumour cells exhibited some irregularity of cytoplasmic staining and of nuclear size.

V. Experiments with partially hepatectomized rats

The response of liver RNA metabolism to diet was examined during liver regeneration after partial hepatectomy, in order to determine whether a state of active cell division in the liver alters its sensitivity to diet.

Male albino rats weighing 200–240 g. were fed for 3 days on the protein-containing synthetic diet described above. On the 4th day, half of the animals were transferred to the synthetic protein-free diet while the other rats continued to eat the protein-containing diet. After the second meal of that day had been fully consumed, half the rats in each dietary group were submitted to partial hepatectomy by the method of Crandall and Drabkin (1946). The remaining rats were subjected to laparotomy and served as control animals. Rats in both the experimental and control groups continued to eat the protein-containing or protein-free diets for a further two days. On the morning of the 3rd day after operation, they were injected with ^{32}P and with [^{14}C]glycine while in the fasting state. Some rats from the hepatectomized and control groups which had received the protein-containing diet were also fed casein just prior to isotope injection. In this way, animals fasting after protein-containing and protein-free diets could be compared with animals actively absorbing amino acids. All animals were killed 6 hours after isotope injection, that is 72 hours after operation. The livers were removed for the measurement of isotope uptake.

RESULTS

Experiments with the transmissible hepatoma

These studies were carried out on rats bearing a transmissible hepatoma implanted subcutaneously. Comparisons were made of the responses of the tumour and of the normal liver of the same animal to variations in the protein content of the diet.

Dietary protein and the composition of hepatoma cells and normal liver cells

In Table I, the protein and RNA content of the hepatoma and of the liver following different diets have been computed per mg. DNAP. The DNA content of the livers of mature animals is a measure of cell number which is unaffected by diet (Thomson, Heagy, Hutchison and Davidson, 1953), and in consequence can be used as a standard of reference in studying changes in other cell constituents. On the protein-deficient diet, the livers of the tumour-bearing rats showed a considerably lower content of protein and of RNA ; this is similar to the response of the livers of normal rats to protein deficiency (Munro, Naismith and Wikramanayake, 1953). In contrast, the amount of protein and of RNA in the hepatoma cell was uninfluenced by the level of protein in the diet.

It is also apparent from Table I that, on each diet, the amount of protein and of RNA/mg. DNAP is considerably less in the case of the hepatoma than in the case of the liver. This comparison of the constituents of the two tissues on the basis of DNA may not be an accurate reflection of differences in cell composition, since some authors have observed that the hepatoma cell nucleus contains less

TABLE I.—*The Influence of Dietary Protein Intake on the Protein and Ribonucleic Acid Content of the Livers and Tumours of Rats Bearing a Transmissible Hepatoma*

The results are the mean data obtained in 6 experiments. Dietary protein had a statistically significant effect on the amounts of protein and RNA in the liver, but no significant action on these constituents of the hepatoma.

Previous diet	State when killed	Protein N (mg./mg. DNAP)		RNA P (mg./mg. DNAP)	
		Liver	Tumour	Liver	Tumour
Protein-free	Post absorptive	63	32	4·1	1·5
Protein-containing	Post-absorptive	83	32	5·3	1·4
Protein-containing	After meal of protein	91	32	5·3	1·6

DNA than does the average liver cell nucleus (Thomson *et al.*, 1953). Examination of three specimens of our transmissible hepatoma gave values of 0·61, 0·54 and 0·55 pg. DNAP/nucleus, whereas liver nuclei from the same animals contained 1·01, 1·06 and 1·00 pg./nucleus respectively, figures which are similar to the values for hepatoma and for normal liver nuclei recorded by Thomson *et al.* (1953). In order to use DNA to compare the composition of hepatoma cells with that of liver cells, it would thus be necessary to multiply the amounts of protein and of RNA/mg. DNAP in the liver by a factor of about 1·8, a ratio which is only approximate because of the difficulty of making representative preparations of hepatoma nuclei. Thus, when this difference in the DNA content of the nuclei of the two tissues is taken into account, the hepatoma cells are seen to contain even less protein and RNA in comparison with the normal liver cells than the data in Table I would indicate.

Uptake of [^{14}C]*glycine by the free amino acids and* ^{32}P *by the inorganic phosphate of the hepatoma and the liver.*

The specific activities of glycine in the free amino acid pool of the liver and the tumour were examined 3 and 6 hours after isotope injection. Table II shows that the specific activities in the liver and tumour were similar in the case of animals fed on the protein-free diet, but the tumour specific activities were somewhat lower in the two groups of rats previously fed on the diet containing protein. If we assume that the loss of radioactivity from the free amino acid pool of each tissue is exponential over the short period of study, we can calculate the fraction of the activity lost per hour between 3 and 6 hours after injection. The values obtained with the liver and the tumour resemble one another closely (Table II); the values obtained with both tissues are similarly affected by diet.

Uptake of ^{32}P by the inorganic phosphate pools of the two tissues showed a much wider divergence (Table II). The specific activities in the tumour were about one-third of those in the liver, and the rates of loss of activity between 3 and 6 hours after injection were less in the case of the tumour.

Diet and the incorporation of precursors into the RNA of the liver and the tumour

In Table III, the uptakes of [^{14}C]glycine and of ^{32}P by the RNA of the liver and the tumour are expressed in relation to the activities of the precursor pools of glycine and of inorganic phosphate in the tissue (relative specific activities).

TABLE II.—*The influence of Diet on the Specific Activity of* [^{14}C]*Glycine in the Free Amino Acid Pool and of* ^{32}P *in the Tissue Inorganic Phosphate of the Livers and Tumours of Rats Bearing a Transmissible Hepatoma**

Time after injection (hours)	Previous diet	Fed after isotopes injected	Specific activity of free glycine: Liver	Specific activity of free glycine: Tumour	Specific activity of inorganic phosphate: Liver	Specific activity of inorganic phosphate: Tumour
3	Protein-free	*Nil*	1828	1765	5450	1460
	Protein-containing	*Nil*	1605	1177	5930	1825
	Protein-containing	Protein	1056	714	4800	1710
6	Protein-free	*Nil*	887	830	3500	1295
	Protein-containing	*Nil*	878	669	2945	1210
	Protein-containing	Protein	513	345	2045	945
Fraction of activity lost /hour†	Protein-free	*Nil*	0·241	0·252	0·148	0·040
	Protein-containing	*Nil*	0·200	0·189	0·234	0·137
	Protein-containing	Protein	0·242	0·244	0·285	0·198

* The data are the mean results obtained in 2 experiments at each time-interval.

† Velocity constant, that is, the difference in $\log_e$ specific activity at the two time-intervals, divided by the time elapsed (3 hours).

The livers of the tumour-bearing animals showed the same sensitivity to dietary protein level which we observed previously in the livers of normal animals (Clark *et al.*, 1957). Liver RNA took up both isotopes extensively when the tumour-bearing animals were fasting after the protein-free diet, whereas there was a reduced uptake of ^{32}P and almost no uptake of [^{14}C]glycine in the case of the rats fasting after the protein-containing diet. Feeding of protein at the time of isotope injection rapidly restored incorporation to a high level. The tumour RNA did not show this sensitivity to dietary protein level. In particular, the low uptake of isotopes observed in the livers of animals fasting after the protein-containing

TABLE III.—*The Influence of Dietary Protein Intake on the Metabolism of Ribonucleic Acid in the Livers and in the Tumours of Rats Bearing a Transmissible Hepatoma**

The data are calculated as relative specific activities

Time after injection (hours)	Previous diet	Fed after isotope injected	Uptake of [^{14}C] glycine†: RNA-adenine: Liver	RNA-adenine: Tumour	RNA-guanine: Liver	RNA-guanine: Tumour	Uptake of ^{32}P‡ by RNA: Liver	Uptake of ^{32}P‡ by RNA: Tumour
3	Protein-free	*Nil*	1·8	6·2	3·6	7·9	4·9	21·0
	Protein-containing	*Nil*	0·4	10·0	0·4	12·4	2·0	18·6
	Protein-containing	Protein	4·5	14·7	6·1	17·6	3·3	21·8
6	Protein-free	*Nil*	9·0	21·3	12·2	21·7	13·2	54·1
	Protein-containing	*Nil*	1·4	30·2	0·5	28·3	6·4	50·3
	Protein-containing	Protein	16·1	38·9	20·6	40·6	11·0	52·3

* Mean data obtained in 2 experiments with [^{14}C] glycine and 3 experiments with ^{32}P.

† The specific activity of the glycine portion of the purine nucleus expressed as a percentage of the specific activity of the free glycine in the tissue.

‡ Specific activity of ribonucleic acid P expressed as a percentage of the specific activity of the inorganic phosphate of the tissue.

diet was not observed in the tumour, and the feeding of protein at the time of isotope injection did not alter ^{32}P incorporation into tumour RNA significantly and produced a relatively small change in uptake of [^{14}C]glycine.

It is also possible to express the data in the form of total uptake per cell, obtained by multiplying the relative specific activities given in Table III by the amounts of RNAP/mg. DNAP (total relative activities). As might be expected from the comparatively small effect of diet on the amount of RNA in the liver cell and the absence of an effect on tumour cell composition (Table I), these total relative activities follow essentially the same pattern as the relative specific activities given in Table III, and have therefore not been tabulated.

A further difference in the behaviour of RNA metabolism in the liver and in the tumour emerges when the ratio of glycine uptake by guanine relative to glycine uptake by adenine is calculated (Table IV). These ratios have been computed from the data given in Table III and show essentially the same picture at 3 and 6 hours after injection of the isotopes. In the case of liver RNA, the uptake of radioactivity by guanine was greater than the uptake by adenine when the rats were receiving the protein-free diet. This ratio was much lower when the rats were fasting after the protein-containing diet, but rose again when protein was fed at the time of isotope injection. These effects of diet on the relative uptakes of [^{14}C]glycine by the guanine and adenine of liver RNA are statistically significant. In contrast, the guanine-adenine ratio of the tumour RNA remains constant on all diets.

TABLE IV.—*The Influence of Dietary Protein Intake on the Relative Uptake of* [^{14}C]*Glycine by the Purine Bases of Ribonucleic Acid in the Livers and in the Tumours of Rats Bearing a Transmissible Hepatoma**

Time after injection (hours)	Previous diet	Fed after isotope injected	Guanine/adenine ratio for uptake of [^{14}C]glycine	
			Liver	Tumour
3	Protein-free	*Nil*	2·1	1·3
	Protein-containing	*Nil*	1·1	1·3
	Protein-containing	Protein	1·5	1·3
6	Protein-free	*Nil*	1·5	1·1
	Protein-containing	*Nil*	0·4	1·0
	Protein-containing	Protein	1·3	1·1

* Mean data obtained in two experiments.

Experiments with intrahepatic tumours

The preceding results show that the response of the transmissible hepatoma to changes in protein intake was different from the response of the liver. In order to establish that these differences between the tumour and the liver were not due to the subcutaneous position of the tumour, a few similar experiments were performed on tumours induced in the liver by feeding dimethylaminoazobenzene to rats. In this case, the response of the tumour and of the non-malignant portions of the liver to diet can be compared under circumstances in which they both receive the same (portal) supply of blood. Fig. 1 shows that, even under these circumstances, the metabolism of RNA in liver tumour cells still differs from that

of normal liver cells. Rats with intrahepatic tumours were injected with ^{32}P and [^{14}C]glycine while fasting after a period of several days feeding on the protein-containing diet or the protein-free diet. In the non-malignant portions of the liver there was the usual *reduced* incorporation of these isotopes when the animals were fasting after a diet containing protein, whereas the RNA of tumours removed from the same livers tended to show a *higher* isotope uptake after the protein-containing diet than after the protein-free diet.

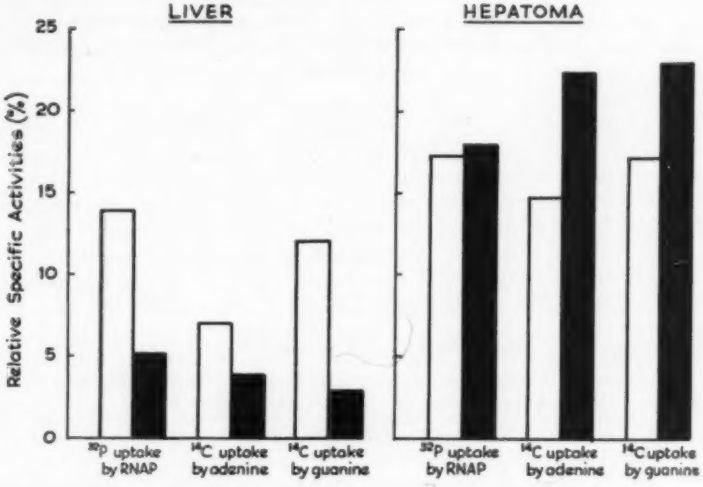

FIG. 1.—Incorporation of ^{32}P and of [^{14}C]glycine into the RNA of tumours induced in the liver by feeding dimethylaminoazobenzene to rats, compared with their incorporation into the RNA of non-malignant portions of the same livers. Animals fasting after several days of feeding with a protein-free diet are shown by open columns; animals fasting after a protein-containing diet are shown by solid columns. The data are the mean results obtained in two experiments in which the rats were killed 6 hours after injection of the isotopes. The results are given as relative specific activities, that is, the specific activity of RNA phosphorus expressed as a percentage of the specific activity of the inorganic phosphate of the tissue, and the specific activity of the glycine portions of RNA adenine and guanine expressed as a percentage of the specific activity of free glycine.

Experiments with partially hepatectomized rats

The sensitivity of the liver to protein level in the diet was studied during regeneration after partial hepatectomy, in order to determine whether the change in RNA metabolism observed in the hepatoma could result from its being in a state of active cell division. Rats were fed on the protein-containing or protein-free diets for the first 72 hours after hepatectomy or laparotomy (control procedure), and then uptake of ^{32}P and of [^{14}C]glycine by the liver RNA was measured. The results (Table V) are expressed in the form of relative specific activities; since the amounts of RNAP/mg. DNAP were not greatly affected by diet, calculation of the data in the form of total isotope uptake/cell (total relative activities) would give essentially the same picture. In both the hepatectomized and the laparotomized series of animals, incorporation was affected in a similar manner

by diet; there was a marked reduction in uptake of both isotopes into RNA when the rats were fasting after the diet containing protein and a restoration of uptake when protein was fed at the time of isotope injection (Table V). Uptake of ^{32}P into liver DNA was considerably elevated in the hepatectomized group, a finding which is consistent with a state of active cell division. It can thus be concluded that an increase in the rate of cell multiplication does not by itself reduce the sensitivity of liver RNA metabolism to dietary protein level.

TABLE V.—*The Influence of Dietary Protein Intake on the Metabolism of Ribonucleic Acid in the Liver During Regeneration After Partial Hepatectomy, Compared with Laparotomy**

Operation	Previous diet	Fed after isotope injection	Mg. RNAP per mg. DNAP	Uptake of [^{14}C] glycine† by RNA		Uptake of ^{32}P‡	
				Adenine	Guanine	RNA	DNA
Hepatectomy	Protein-free	*Nil*	3·8	7·9	18·4	15·1	4·9
	Protein-containing	*Nil*	3·6	0·8	1·0	5·9	2·3
	Protein-containing	Protein	3·8	12·1	18·8	14·7	3·4
Laparotomy	Protein-free	*Nil*	3·1	4·7	6·7	9·8	0·9
	Protein-containing	*Nil*	3·5	0·3	0·2	4·8	0·8
	Protein-containing	Protein	3·4	7·2	11·2	8·8	0·8

* The data are the mean results obtained in two experiments, in which the rats were killed 6 hours after injection of the isotopes.

† The specific activity of the glycine portion of the purine nucleus expressed as a percentage of the specific activity of free glycine in the tissue (relative specific activity).

‡ The specific activity of ribonucleic acid or deoxyribonucleic acid P expressed as a percentage of the specific activity of the inorganic phosphate of the tissue (relative specific activity).

DISCUSSION

The response of liver RNA metabolism to changes in protein intake has been described in a previous paper (Clark *et al.*, 1957). Compared with rats fasting after receiving a protein-free diet, animals fasting after a diet containing protein were observed to incorporate less ^{32}P and very much less [^{14}C]glycine into liver RNA. Incorporation levels in these animals were dramatically raised when protein was fed at the time of isotope injection. A considerable degree of sensitivity of liver RNA metabolism to the available supply of amino acids was thus established.

In the present series of experiments, we have investigated the question of whether a tumour derived from liver cells would still exhibit fluctuations in RNA metabolism with changes in protein intake or whether its metabolic activities would be less dependent on dietary conditions. For our main series of experiments a transmissible hepatoma implanted subcutaneously was preferred to the use of intrahepatic tumours produced by feeding carcinogenic azo-dyes, because the type of tumour produced in the latter way cannot be controlled (Edwards and White, 1941), and furthermore the distinction between malignant and non-malignant parts of the liver sometimes presents difficulties. When the protein intake of rats bearing the transmissible hepatoma was varied, liver RNA showed the same sensitivity to diet observed previously in the case of normal rats, but the tumour did not show this response. The protein and RNA contents of the tumour cell were unaffected by the level of protein intake (Table I) and the uptake of

isotopes by the RNA of the tumour (Tables III and IV) also did not respond in the characteristic manner of liver cells.

Certain possible reasons for these differences between the tumour and the liver can be excluded. In the first place, the tumour was supplied by the systemic circulation whereas the liver received portal blood, and this might be the reason why the liver alone was affected by the supply of amino acids coming from the diet. However, this explanation was eliminated by a few experiments carried out on hepatomas which had been induced within the liver by feeding dimethylaminoazobenzene. These intrahepatic tumours were also found to be insensitive to dietary changes which caused the usual alterations in RNA metabolism in adjacent non-malignant areas of the same livers (Fig. 1). A second possibility is that the tumour was insensitive to diet because it was tissue in a state of active cell division. A study of the effect of dietary protein level on RNA metabolism in regenerating liver (Table V) showed, however, that stimulation of cell division in the liver does not by itself affect the capacity of the liver cell to respond to the protein content of the diet. Consequently, the peculiarities of RNA metabolism in the hepatoma are not simply the result of rapid cell division.

The evidence thus points to a fundamental difference between the metabolism of RNA in the hepatoma cell and in the normal liver cell, RNA metabolism in the hepatoma having lost its capacity to respond to changes in dietary protein level which affect RNA metabolism in normal hepatocytes. In order to offer an explanation of the insensitivity of the tumour cell to diet, it is necessary to consider first the mechanism by which the RNA of normal liver cells responds to the protein content of the diet. In our previous study (Clark *et al.*, 1957) we reached the conclusion that the supply of amino acids coming to the liver influenced the rate of RNA breakdown. The striking effect which protein intake has on the uptake of isotopes by liver RNA was attributed by us to differences in the extent to which RNA breakdown products dilute isotopic labelling in the pools of RNA precursor compounds. Some indication has been obtained by cell fractionation procedures (Wikramanayake, Heagy and Munro, 1953) of the intracellular location of the RNA which is affected in this way by dietary protein level. Rats receiving a protein-free diet showed a loss of RNA from the liver which was essentially confined to the microsomal fraction of the cell precipitated by centrifuging at a comparatively low speed (18,000 *g*. with 0·25 M. sucrose homogenates). The sensitivity of RNA metabolism to dietary protein level thus appears to be associated with changes in this cell fraction. Microsome fractions sedimented from liver and other tissues at these relatively low speeds contain RNA in the form of ribonucleoprotein particles attached to small vesicles (Palade and Siekevitz, 1956 ; Douglas and Munro, 1959) which are derived from the endoplasmic reticulum of the intact cell (Palade and Siekevitz, 1956). Loss of RNA from this fraction should therefore be represented in the intact liver cell by changes in the endoplasmic reticulum. Two independent studies of liver cells with the electron microscope (Fawcett, 1955 ; Bernard and Rouiller, 1956) have in fact shown that the endoplasmic reticulum is greatly reduced or even absent after several days of fasting and reappears within a few hours of giving a meal rich in protein. A diet low in protein is much less effective in causing restoration of the reticulum (Fawcett, 1955). It can be concluded that these losses and gains in endoplasmic reticulum, with its adherent ribonucleoprotein particles, account for the sensitivity of RNA metabolism in the liver to changes in protein intake.

In view of these conclusions about the part played by the endoplasmic reticulum in the RNA metabolism of the normal liver cell, it is significant that the Novikoff transmissible hepatoma (Novikoff, 1957) is deficient in a particulate fraction which corresponds approximately to the microsome fraction discussed above. Furthermore, it has been observed by Howatson and Ham (1955) with the electron microscope that liver tumour cells have only a scanty endoplasmic reticulum. The failure of RNA metabolism to be affected by dietary protein level in the case of hepatomas is thus likely to be related to the inability of this cell to form endoplasmic reticulum.

SUMMARY

1. Rats with a transmissible hepatoma implanted under the skin were given diets containing different amounts of protein and the metabolism of RNA in the liver and in the tumour were compared.

2. When the animals were fed on a diet deficient in protein, the liver cells lost protein and RNA, but the composition of the tumour cells remained unaltered.

3. When protein intake was varied, considerable changes in the incorporation of [^{14}C]glycine and of ^{32}P into the RNA of the liver were observed, but the RNA of the tumour did not show these changes in response to dietary variations.

4. The possibility that the subcutaneous position of the hepatoma might be responsible for its lack of sensitivity to diet was excluded by experiments which showed the same insensitivity to diet in tumours produced in the liver by feeding a carcinogenic diet. Studies on livers regenerating after partial hepatectomy showed that active cell multiplication does not alter the response of RNA metabolism to diet from that observed in the case of the resting liver cell. Consequently, the abnormal pattern of RNA metabolism observed in the tumour is not simply that of a growing tissue.

5. Evidence is presented to show that the insensitivity of RNA metabolism in the normal liver cell to dietary protein level is dependent on changes in the amount of endoplasmic reticulum, and that the insensitivity of the hepatoma cell to diet is due to its inability to form endoplasmic reticulum.

Part of the work described in this paper was carried out by Dr. Clark during her tenure of a Barbour scholarship awarded by the University of Glasgow. Dr. K. S. Kirby of the Chester Beatty Research Institute, London, most generously supplied us with samples of the transmissible hepatoma and with the August strain of rats. Mr. D. Goldberg carried out exploratory studies which greatly assisted us in planning the experiments on hepatectomized rats. Histological sections of the tumours were prepared under the direction of Dr. H. S. D. Garven, to whom we are most grateful. We also wish to acknowledge with gratitude grants for expenses and for scientific assistance to one of us (H.N.M.) from the Medical Research Council.

REFERENCES

Bernard, W. and Rouiller, C.—(1956) *J. biophys. biochem. Cytol.*, Suppl. 2, 73.

Clark, C. M., Naismith, D. J. and Munro, H. N.—(1957) *Biochim. biophys. Acta*, **23**, 587.

CRANDALL, M. W. AND DRABKIN, D. L.—(1946) *J. biol. Chem.*, **166**, 653.
DAOUST, R. AND HOOPER, C. E. S.—(1957) *Canad. J. Biochem. Physiol.*, **35**, 721.
DOUGLAS, T. A. AND MUNRO, H. N.—(1959) *Exp. Cell Res.*, **16**, 148.
EDWARDS, J. E. AND WHITE, J.—(1941) *J. nat. Cancer Inst.*, **2**, 157.
FAWCETT, D. W.—(1955) *Ibid.*, **15**, 1475.
HOWATSON, A. F. AND HAM, A. W.—(1955) *Cancer Res.*, **15**, 62.
MILLER, J. A., KLINE, B. E. AND RUSCH, H. P.—(1946) *Ibid.*, **6**, 674.
MUNRO, H. N. AND CLARK, C. M.—(1958) *Proc. Nutr. Soc.*, **17**, xxiv.
Idem AND NAISMITH, D. J.—(1953) *Biochem. J.*, **54**, 191.
Idem, NAISMITH, D. J. AND WIKRAMANAYAKE, T. W.—(1953) *Ibid.*, **54**, 198.
NOVIKOFF, A. B.—(1957) *Cancer Res.*, **17**, 1010.
PALADE, G. E. AND SIEKEVITZ, P.—(1956) *J. biophys. biochem. Cytol.*, **2**, 171.
SMELLIE, R. M. S., HUMPHREY, G. F., KAY, E. R. M. AND DAVIDSON, J. N.—(1955) *Biochem. J.*, **60**, 177.
THOMSON, R. Y., HEAGY, F. C., HUTCHISON, W. C. AND DAVIDSON, J. N.—(1953) *Idem*, **53**, 460.
WIKRAMANAYAKE, T. W., HEAGY, F. C. AND MUNRO, H. N.—(1953) *Biochim. biophys. Acta*, **11**, 566.

EFFECT OF CARCINOGENS AND RELATED COMPOUNDS ON THE GROWTH OF EHRLICH ASCITES CARCINOMA AND ITS POSSIBLE MECHANISM

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A GROWTH promoting substance has recently been isolated from animal tissues and egg-yolks which markedly enhances the growth of transplantable tumours by stimulating the biosynthesis of proteins (Hradec, 1958*a*). Some chemical as well as biological properties of this factor indicate its possible relation to an endogenous carcinogenic substance (Kleinenberg, Neufach, and Shabad, 1941). For this reason it was necessary to evaluate the possible effect of chemical carcinogens on the growth rate of experimental tumours.

On the basis of some older papers a general growth promoting activity of carcinogenic hydrobarbons can be assumed. Enhanced growth was observed in plants when higher doses of 3,4-benzpyrene were added (Rarei and Gummel, 1938) the same being true for regeneration in worms when the same substance or 20-methylcholanthrene was used (Owen, Weiss, and Prince, 1939). Similar effects were shown when these substances were tested in tissue cultures. Very small doses of carcinogenic hydrocarbons exhibited a stimulating effect on the culture growth while higher doses led to an inhibition of this process (Creech, 1940).

On the other hand, an inhibitory effect of 3,4-benzopyrene on the growth of a transplantable tumour in rats was established when this substance was given in daily injections to tumour-bearing animals (Haddow, 1935). This effect was claimed to be specific for various carcinogenic substances while the majority of non-carcinogenic compounds were without effect (Euler and Skarzynski, 1942).

No definite conclusions can therefore be drawn from these experiments, some of them showing an enhancing effect of carcinogenic substances on the tissue growth while others on the contrary demonstrating an inhibitory activity of these substances. It seems probable that the choice of diverse experimental techniques as well as doses of substances used in these experiments may be responsible for these conflicting results.

We thought it necessary therefore to test the effect of a wide range of doses of carcinogenic substances as well as of some chemically related non-carcinogenic compounds on the growth rate of Ehrlich ascites carcinoma in mice. When trying to explain the possible mechanism of the stimulating activity of some of these substances the probable effect on the biosynthesis of proteins could not be excluded. It is a well established fact that some substances affecting the growth of experimental tumours also have a marked effect on the incorporation of labelled amino acids into proteins. This is true for instance for sarcomycin (Quastel, 1958). No experiments have so far been reported on the effect of polycyclic hydrocarbons

and related compounds on the incorporation of labelled amino acids into proteins. Evidence is presented that the stimulatory effect on the biosynthesis of proteins promoted by these substances may be related to the enhanced tumour growth and that this effect should not be omitted when attempts are made to explain the mode of action of chemical carcinogens.

MATERIALS AND METHODS

Experimental animals

White albino mice of mixed stock or inbred rats of the Wistar strain were used throughout our experiments. Animals weighing 20–25 g. and 180–200 g., respectively, of both sexes, housed in glass cages, were fed Larsen diet and given water *ad libitum*.

In vivo *procedures*

Each dose of substance tested was usually administered to a group of 30 mice. One experimental series, used for testing the effect of one particular substance in different amounts, consisted of 6–10 such groups together with one control group.

Compounds used in these experiments, i.e. 3,4-benzopyrene, 1,2,5,6-dibenzanthracene, anthracene, phenanthrene, chrysene, cholesterol, and dehydrocholic acid, were all of C.P. grade and no further purification of them was performed. For administration to animals they were finely homogenized in olive oil in a glass homogenizer and administered in one subcutaneous injection (0·1 ml.) on the day preceding the inoculation of Ehrlich ascites carcinoma. Pure olive oil was administered in the same way to control animals.

Ehrlich ascites carcinoma was maintained in the same stock of mice. For the purposes of transplantation, 5–10 tumour-bearing animals were sacrificed, ascites drawn, pooled, diluted with saline to contain $3–5 \times 10^6$ cells in 0·1 ml., and administered intraperitoneally, 0·1 ml. each, to all mice of one experimental series. No attempts were made to give exactly the same number of tumour cells to different experimental series since control groups were included in all of them.

The number of dead mice was determined at the same time each day. From these data the mean survival time in days after transplantation of the tumour was computed for each experimental group and its significance when compared with the control group tested by *t* test.

In vitro *procedures*

Pooled Ehrlich ascites from several mice was preserved in small portions in a freezing unit (−30° C.). It was found, that the ability of this material to incorporate a labelled amino acid was not significantly decreased after 30 days of preservation. For *in vitro* experiments, the ascitic fluid was quickly thawed by immersing in hot water, diluted with a solution of isotonic KCl and $KHCO_3$ (1000 : 8) till the protein content of the suspension was 0·5–0·7 per cent (the same dilution being then used for the whole batch of Ehrlich ascites on alternate days) and, after addition of labelled amino acids and substances to be tested, immediately incubated.

For some experiments, 5 per cent homogenates of liver and spleen tissue of rats were prepared in a glass homogenizer. Animals were sacrificed by a blow on the head, tissues were immediately excised, minced with scissors, and homo-

genized in an ice cold solution of isotonic KCl and $KHCO_3$ in a Potter-Elvehjem homogenizer cooled with ice cold water for 2–3 minutes.

DL-[^{35}S]Methionine with an average activity of 0·6–0·7 mc per mg., was obtained from the Radiological Research Institute in Prague. A solution of 1·5 mg. of the amino acid in 100 ml. of the above medium was used.

[Carboxy-^{14}C]Glycine, with an activity of 0·16 mc per mg., was obtained from the same source, was used in some experiments. A solution of 26 mg. of this compound in 10 ml. of incubation medium was used.

Compounds used in these experiments, except of those already stated, were 20-methylcholanthrene, pyrene, deoxycholic acid, and sodium glycocholate, all of C.P. grade not further purified. Substances were finely homogenized in olive oil and the resulting suspension or solution added to the incubation mixture. Pure olive oil was given to control samples.

All control as well as experimental specimens were incubated in triplicate samples. Each sample in a conventional Warburg flask containing 2 ml. of diluted ascites tumour, or liver or spleen homogenate, 0·1 ml. of [^{35}S]methionine or in some instances glycine, and 0·1 ml. of olive oil or suspension of tested substance was incubated with shaking in a Warburg apparatus at 37·5° C. for 45 minutes.

After this, duplicate samples (0·5 ml.) from each flask, were precipitated with 1 ml. of 20 per cent trichloroacetic acid. The precipitate was centrifuged after 10 minutes, homogenized in a glass homogenizer in 2 ml. of 5 per cent trichloroacetic acid, and centrifuged again. It was then successively washed in the same way with another 2 ml. of 5 per cent trichloroacetic acid, hot (90° C.) 5 per cent trichloroacetic acid (2 ml.), twice with 96 per cent ethanol (2 ml.) and once with 2 ml. ethanol-ether mixture (1 : 1). The precipitate was then finely homogenized in ether and samples for measurement for radioactivity were prepared on weighed filter-paper discs. Details of this procedure as well as those used for radioactivity assay are given in our recent paper (Hradec, 1958*b*).

Duplicate controls at zero time incubation were done with each series. Net specific radioactivity obtained by subtracting the mean value of the control sample from those incubated was taken into account in evaluation of the experiments. Standard errors not exceeding $\pm$ 3 per cent for duplicate samples of the same flask and $\pm$ 5 per cent for average values from the same three specimens, were found in the majority of our experiments.

In the case of Ehrlich tumour, the actual specific radioactivities were 121–243 counts/minute per mg. of dried protein precipitate in various batches of this ascites tumour. When using the liver or spleen homogenate, actual specific activities were 25 to 42 counts/minute per mg. of dried protein precipitate. Samples weighing 1·8–3·6 mg. were usually counted.

In some of our experiments the method of Melchior and Halikis (1952) was used for the isolation of proteins for radioactivity assay to exclude the possible occlusion of radioactivity on protein precipitates which might cause some sources of errors in our original method.

RESULTS

Effect of polycyclic hydrocarbons and related compounds on the survival rate of mice bearing the Ehrlich carcinoma

The growth of Ehrlich ascites carcinoma in mice after administration of various compounds tested is given in Table I.

TABLE I.—*Effect of Carcinogenic Hydrocarbons and Related Compounds on the Survival Rate of Mice Bearing the Ehrlich Ascites Carcinoma*

Compound	Survival time	Dose (10^{-n} mg.) 0	1	2	3	4	5	6	7	8	C
3,4-benzopyrene	mean	14·9	16·3	15·5	16·1	15·5	13·8	12·5	12·9	13·3	14·1
	ranges	12–18	11–20	12–19	13–19	13–17	10–18	10–15	9–15	11–16	12–19
	S	2·18	2·88	2·16	1·75	1·17	1·58	1·14	1·48	1·26	2·07
	P	0·2	0·01	0·01	0·01	0·01	0·1	0·01	0·01	0·01	—
1,2,5,6-dibenzanthracene	mean	14·0	13·3	13·9	12·8	12·4	13·2	13·6	—	—	13·2
	ranges	12–17	10–15	10–17	10–16	10–15	10–15	9–18	—	—	10–15
	S	1·34	1·17	1·76	1·16	1·42	1·34	2·18	—	—	1·22
	P	0·01	—	0·1	0·05	0·01	—	0·2	—	—	—
Anthracene	mean	14·9	15·3	16·4	15·3	12·9	13·7	14·2	14·4	14·3	14·1
	ranges	11–17	10–18	12–21	13–17	10–15	12–19	12–20	11–17	11–17	12–19
	S	1·25	1·78	2·86	1·29	1·22	1·57	1·47	1·62	1·84	2·07
	P	0·1	0·02	0·01	0·01	0·01	0·05	—	—	—	—
Phenanthrene	mean	14·5	14·1	13·7	14·5	14·4	14·0	14·1	—	—	14·3
	ranges	14–20	10–17	12–17	8–17	8–18	9–17	8–18	—	—	9–17
	S	2·64	2·12	1·60	2·31	2·25	1·96	2·16	—	—	1·84
	P	—	—	—	—	—	—	—	—	—	—
Chrysene	mean	11·8	12·4	12·5	11·8	12·3	13·1	12·0	12·7	12·0	14·3
	ranges	7–14	7–14	7–16	10–13	8–14	11–14	10–14	9–15	11–14	10–16
	S	1·79	1·78	2·06	0·30	1·67	0·28	1·27	1·63	0·31	1·96
	P	0·01	0·01	0·01	0·01	0·01	0·01	0·01	0·01	0·01	—
Cholesterol	mean	11·8	13·1	12·6	12·2	12·6	12·8	13·2	—	—	13·1
	ranges	9–15	9–20	9–19	8–18	9–17	9–20	10–19	—	—	10–20
	S	1·67	2·88	2·87	2·78	2·64	2·88	2·68	—	—	2·90
	P	0·05	—	0·8	0·3	—	—	—	—	—	—
Dehydrocholic acid	mean	15·1	15·1	15·4	16·1	14·3	14·0	12·0	11·8	11·8	13·5
	ranges	10–17	11–17	13–18	9–18	9–16	9–16	9–16	9–15	9–17	9–16
	S	2·00	2·19	0·32	2·17	1·95	2·27	2·40	2·34	2·11	1·93
	P	0.01	0.01	0.01	0.01	0.2	0.4	0.05	0·01	0·01	—

All values are given in days after the transplanatation of the Ehrlich tumour.
C = control group.
S = standard deviation.

Phenanthrene in dose levels 1–0·000001 mg. proved of no effect on the survival time of mice with the tumour. Little effect was also observed when cholesterol was used. Only the highest dose, i.e. 1 mg., caused a slight enhancement of the tumour growth.

A marked change in the survival rate of tumour-bearing mice was found after the administration of both strong carcinogenic agents, benzopyrene and dibenzanthracene. High doses of these substances seem generally to possess an inhibiting effect while trace doses of them caused a marked acceleration in tumour growth. The highest dose used, i.e. 1 mg., proved to be ineffective in the case of benzopyrene while the next lower dose reduced significantly the growth rate of the tumour. Trace doses of this compound exhibit a pronounced suppressing effect on the survival time of animals bearing the Ehrlich tumour. Very similar effects were seen when dibenzanthracene was used. Higher doses of this substance,

however, seem to be necessary for the inhibition as well as stimulation of the tumour growth as if the same pattern of effectiveness were being postponed to higher dose levels.

Unexpectedly, the same general pattern was found when the non-carcinogenic anthracene was used. There seems to be only one difference when compared with the effect of carcinogenic hydrocarbons. Intermediate doses which are without effect are not found between inhibitory dose levels and those which enhance. The amount of this compound necessary for increasing the mean survival time of

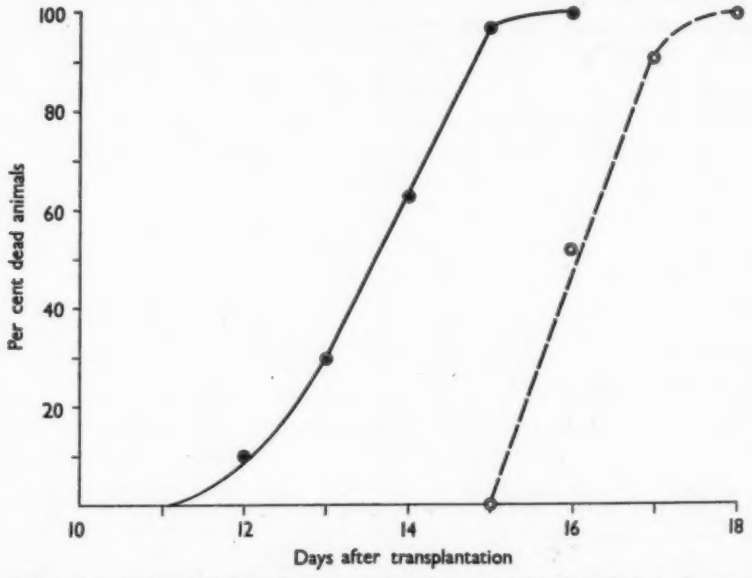

FIG. 1.—Mortality of mice administered 0·001 mg. dehydrocholic acid (dashed line) when compared with that of controls (full line).

animals corresponds roughly to that of benzopyrene while higher doses are required for enhancement of tumour growth.

Similar effects to those obtained in the case of carcinogenic hydrocarbons and anthracene are also found when dehydrocholic acid is used. There was, however, a unique position of this substance among all other compounds tested. High doses increasing the mean survival time of tumour-bearing mice had a much more pronounced effect than carcinogenic hydrocarbons in the same doses. In many groups receiving these doses of dehydrocholic acid all animals were living at the time when all controls had died, as is seen in Fig. 1.

In other respects, however, there is little difference when using this compound or benzopyrene. The same trace doses enhance the tumour growth, these being separated by ineffective doses from those which inhibit.

A striking effect was observed when chrysene was used. This compound was the only one of all substances tested which caused an enhancement of tumour

growth in all doses used, the same effect being observed after administration of 1 mg. of this substance as in the case of 0·00001 μg.

Effect of polycyclic hydrocarbons and related compounds on the incorporation of labelled amino acids into proteins

Incorporation of [^{35}S]methionine into the proteins of Ehrlich ascites carcinoma *in vitro* is affected by the addition of some polycyclic hydrocarbons or related compounds to the incubation mixture, as is seen from Table II.

TABLE II.—*Effect of Chemical Carcinogens and Related Compounds on the Incorporation of* [^{35}S]*Methionine into the Proteins of Ehrlich Ascites Carcinoma* In Vitro (*given as per cent of stimulation or inhibition if control specimen's value is equal to* 100 *per cent*)

Compound	Dose (10^{-n} mg.)								
	0	1	2	3	4	5	6	7	8
Benzopyrene	−66	−22	−17	+23	0	0	− 6	+72	+27
Dibenzanthracene	− 7	−24	+41	+140	+91	+164	−63	+ 3	− 7
Methylcholanthrene	+67	+38	+41	+98	+46	+123	+28	−23	0
Anthracene	+22	+23	+18	+20	+19	+23	+ 1	0	0
Phenanthrene	−24	−18	−16	−13	−11	− 8	0	0	0
Pyrene	−57	−36	−31	−20	−19	−19	0	0	0
Chrysene	−10	−17	−17	+25	+30	+22	−12	−22	−15
Cholesterol	−24	−19	−20	−22	−14	−10	− 6	0	0
Deoxycholic acid	−61	−24	−25	+ 8	+23	+15	−14	0	0
Dehydrocholic acid	−67	−38	−22	−15	−14	−11	−11	0	0
Sodium glycocholate	−19	−16	−14	− 8	− 5	0	0	0	0

Carcinogenic substances seem to be able to effect the incorporation of labelled methionine in both directions—some doses of them inhibit this process partially while others stimulate it. This effect is very clearly seen especially when strong carcinogenic hydrocarbons, i.e. benzopyrene, dibenzanthracene, or methylcholanthrene, were added to the incubation medium. The general dose-response pattern is very similar in all these compounds. It consists of two well defined doses of maximal stimulating effect. The first of them, common for all three compounds tested, is 1 μg., while the other, for dibenzanthracene and methylcholanthrene only, seems to be 0·01 μg. This second effective dose is also present in the case of benzpyrene, here it lies, however, in significantly lower dose levels, i.e. 0·0001 μg. On the contrary, such well defined doses of maximal inhibitory effect are not found when carcinogenic compounds are used. High doses of benzopyrene and dibenzanthracene depressed the incorporation of labelled methionine into the proteins of Ehrlich ascites carcinoma at a dose of 1 or 0·1 mg.; no similar effect was found, however, when methylcholanthrene was used. On the contrary, high doses of this compound stimulate this process to a considerable extent. It cannot be excluded, however, that even higher doses than those used would be necessary to exhibit the depressing effect of methylcholanthrene.

Trace doses lower than those showing the second stimulation maximum, seem also to be capable of inhibiting partially the incorporation of labelled methionine into proteins. This is seen in the case of dibenzanthracene and methylcholanthrene in dose levels of 0·001 and 0·0001 μg., respectively. The same inhibiting dose was found in the case of benzopyrene. Doses of highest stimulating effect are sepa-

rated by dose levels with somewhat lower stimulating activity or none at all (as in the case of benzpyrene).

A different pattern was found when weak carcinogenic substances, chrysene and deoxycholic acid, were used instead of the strongly carcinogenic ones. A similar general dose response is seen, some doses exhibiting a depressing while others a stimulating effect. Only one highest effective dose is demonstrable, however, in this case. It seems to be the same with both substances tested, i.e. 0·1 μg. It is definitely different from both maximally effective doses found when strong carcinogens were used. Two suppressing dose levels were observed, the first in high doses while the other in lower ones. Similarly, as in the case of strong carcinogens, trace doses have an inhibiting activity when using weak carcinogens. It seems necessary, however, for these doses to be somewhat higher than in the case of strong carcinogens.

Non-carcinogenic hydrocarbons, cholesterol, and some bile acids, with the exception only of anthracene, had no stimulating effect on the incorporation of labelled methionine into the proteins of Ehrlich ascites carcinoma. High doses of these compounds have a definite inhibiting effect on this process, this effect gradually diminishing when lower doses are used.

Anthracene possesses a unique position in the whole group of substances tested. The addition of this compound to the incubation mixture markedly stimulates the incorporation. No alternate stimulating and inhibiting effect is demonstrable, however, as in the case of carcinogens ; this action seems to be largely dose-independent, and the same effect is seen at various dose levels from 1 mg. to 0·01 μg.

Since high blank values (10–15 per cent of the incubated samples values) were found when using our original method of preparing protein samples for radioactivity assay it was necessary to exclude the possible effect of occlusion of radioactivity on the protein precipitates. For this reason the method of Melchior and Halikis (1952) was used in several experiments to exclude this possible source of error. A triplicate precipitation and solution of the precipitated protein effectively removed essentially all the adsorbed radioactivity and no activity was found in zero time controls. The results of experiments performed in this way agreed strictly with those obtained by our original method.

In some of our experiments glycine was used instead of methionine as the labelled amino acid. Closely related results to those obtained with methionine were encountered. This is clearly shown in Fig. 2, where a very similar effect is seen of 3,4-benzopyrene on the incorporation of [^{35}S]methionine and [^{14}C]glycine, respectively, into the proteins of Ehrlich ascites carcinoma.

When the incorporation of labelled methionine into the proteins of liver and spleen homogenate was studied, a very low activity of this process, only 10–20 per cent of that in Ehrlich carcinoma, was found the zero time controls having 60–65 per cent of the radioactivity of incubated samples. Since the zero time controls were very high in these cases, some doubt could be thrown on the significance of these results. For this reason, several experiments were made in the same way and a statistical treatment of their results was performed by means of t test. Highly significant differences were found between various dose levels. The effect of various doses of 1,2,5,6-dibenzanthracene on the incorporation of labelled methionine into the proteins of liver and spleen homogenate is given in Fig. 3. This figure represents average values of 5 experiments done on the same sort of material.

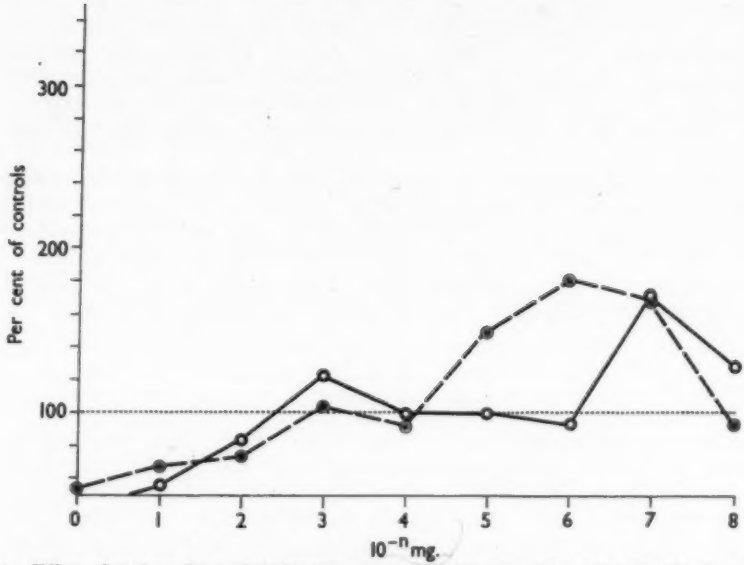

FIG. 2.—Effect of various doses of 3,4-benzopyrene on the incorporation of [^{35}S]methionine (full line) and [^{14}C]glycine (dashed line) into the proteins of Ehrlich carcinoma.

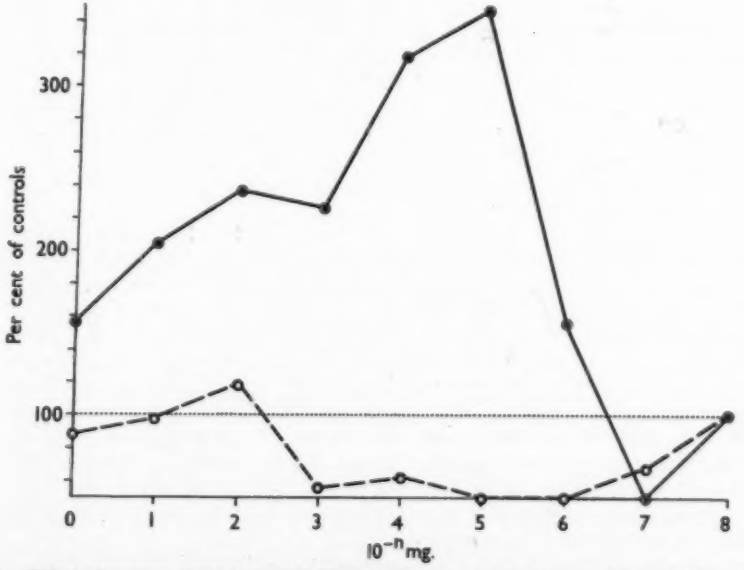

FIG. 3.—Effect of various doses of 1,2,5,6-dibenzanthracene on the incorporation of [^{35}S]methionine into the proteins of liver (full line) and spleen homogenate (dashed line).

When liver homogenate is used, the same general pattern as in the case of Ehrlich ascites carcinoma is obtained. Two doses causing maximum of stimulation are again separated by a less active dose. The second maximally effective dose corresponds well with that described in the case of Ehrlich carcinoma, the first being postponed to higher dose levels. An inhibiting dose could be demonstrated in very low dose levels again closely resembling the results obtained with the tumour.

A clearly different pattern, however, was observed when spleen homogenate was used. The general dose-dependent effect is visible in this case, too, some doses having a stimulating while others an inhibiting effect on the incorporation of labelled methionine into proteins. Only one dose showed a stimulating activity, this being distinctly lower, however, than in the case of liver or tumour tissue. The majority of doses inhibited the incorporation of labelled methionine.

DISCUSSION

Our results clearly demonstrate that higher doses of strongly carcinogenic hydrocarbons show an inhibiting effect on the growth of Ehrlich carcinoma while smaller doses have a quite diverse activity. The diversity of results of the previous authors mentioned already can therefore be explained on the basis of different doses used in their experiments. This changing of stimulating and inhibiting effect is not specific, however, for carcinogenic hydrocarbons, the same activity being demonstrable for instance in the case of non-carcinogenic anthracene and dehydrocholic acid. Thus no real correlation between carcinogenity and the effect of tested compounds on the survival rate of tumour-bearing mice is seen in our experiments.

A similar dose-dependent effect was also seen in our recent experiments, when homogenates of various tissues from tumour-bearing animals were administered to rats before the transplantation of Walker 256 carcinoma took place (Hradec, Dušek, Trojan and Ptáček, 1958). The presence of a similar substance in these tissues can be assumed on the basis of these experiments. Such a compound has since been isolated from this material (Hradec, 1958c).

A very interesting effect was exhibited by chrysene which is known to be a weak carcinogen (Hartwell, 1951).

Our method used for measuring the growth of Ehrlich ascites tumour by survival time of the animals is of course only an indirect criterion for this process (Klein and Révész, 1953). There seems to be no strict correlation between the number of tumour cells and the survival rate of the mice bearing ascites tumours (Patt and Blackford, 1954). On the other hand, the same material was used for the transplantation of all experimental as well as control animals and statistically significant differences in the survival time were found in many instances. It cannot be excluded, however, that some conditioning of the animals occurred due to the action of tested compounds resulting in prolongation of their lives, the actual tumour growth being not affected. This possibility, nevertheless, seems not to be very probable, since cell counts and measurements of the amount of ascites fluid made occasionally in all groups of animals showed no correlation between the cell counts and time of death.

Some similarities are found when comparing the results of our *in vivo* experiments with those obtained *in vitro*. A common general pattern is seen in many

instances. Substances stimulating the incorporation of labelled amino acids into proteins invariably enhance the growth of the Ehrlich tumour *in vivo*. Moreover, higher doses of carcinogenic hydrocarbons show an inhibitory activity in both *in vivo* and *in vitro*, trace doses of these substances being active in the inverse sense. Only in the case of anthracene was this proved not to be so. While *in vivo* results show a close correlation with those obtained when testing strong carcinogens, the effect *in vitro* was always to stimulate the incorporation of labelled amino acid, no dose showing an inhibitory activity comparable with that demonstrable *in vivo*. Another discrepancy between *in vivo* and *in vitro* patterns is found in the case of chrysene. An overall enhancing effect on the growth rate of the Ehrlich carcinoma is dissimilar to the dose-dependent inhibiting or stimulating activity obtained *in vitro*.

A good correlation between the results of both procedures is seen when using cholesterol. Dehydrocholic acid—the most active substance in depressing the incorporation *in vitro* of all substances tested in our experiments—prolongs significantly the survival of mice bearing the Ehrlich tumour.

Since [^{35}S]methionine was used in our experiments it is not posssible to conclude that incorporation of radioactivity into protein was necessarily due to a peptide linkage of methionine. For example cystine can be formed from methionine during the incubation and this could be incorporated into the protein (Melchior and Tarver, 1947). Our results, however, cannot merely be due to the effect of various substances on the rate of formation of such disulphide bonds in proteins since the same results were found when [^{14}C]glycine was used. No other types of bonds than peptide can be assumed when this amino acid is used and it seems quite reasonable to suppose on the basis of our experiments that the tested compounds affect the growth rate of tumours by acting on the biosynthesis of proteins. Additional mechanisms are without doubt involved in the processes as can be assumed from some discrepancy between the *in vivo* and *in vitro* results.

In contrast to the results obtained *in vivo*, the effect of various compounds *in vitro* is quite consistent with their carcinogenic activity. There seems to be a pattern characteristic for carcinogenic substances as well as for the non-carcinogens. The stimulating activity alone, however, seems to be not specific for carcinogenic substances, as the same effect is seen in the case of the non-carcinogenic anthracene. The alternating enhancing and inhibitory effect, however, is demonstrable only when carcinogens are used. In the case of strongly carcinogenic hydrocarbons—benzopyrene, dibenzanthracene, and methylcholanthrene—two definite doses of maximal stimulating activity are seen with a dose of significantly lower enhancing effect lying between both of them. In the case of weak carcinogens, chrysene and deoxycholic acid (Schabad, 1945), only one dose of highest activity was shown. The inhibitory effect of very high as well as of very low doses, the same as with strong carcinogens, is demonstrable. Although in explaining the inhibitory activity of the high doses some unspecific mechanisms must be without doubt taken in account, the inhibitory effect of trace doses seems to be specific for this group of substances.

Non-carcinogenic substances showed in high doses an inhibitory effect on the incorporation of labelled methionine or glycine into proteins, this effect gradually diminishing with the dose used, the smallest doses being without effect.

A unique position in the group of non-carcinogenic substances tested is held by anthracene. Only this compound showed a small, nevertheless significant,

stimulating activity in high doses *in vitro*. It is not possible so far to explain this fact until the mechanism of this stimulating effect is further elucidated.

Three specific patterns therefore seem to be demonstrable when strong carcinogens, weak carcinogens, and non-carcinogenic compounds are tested, as given in Fig. 4.

The study of the effect of carcinogenic substances on the metabolism of proteins seems to be a new, promising approach to the research on the mode of action of these compounds. It will be necessary to make clear the mechanism of this

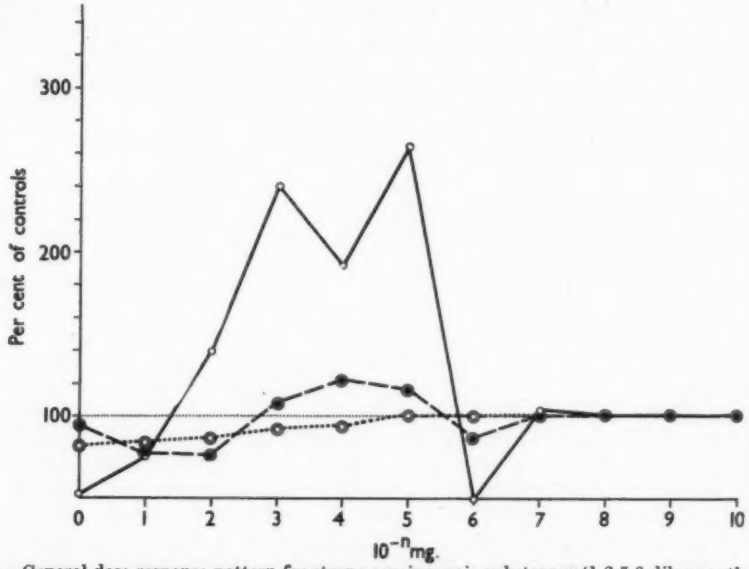

FIG. 4.—General dose response pattern for strong carcinogenic substances (1,2,5,6-dibenzanthracene, full line), weak carcinogens (chrysene, dashed line), and non-carcinogenic compounds (cholesterol, dotted line).

process. It cannot be assumed from our present experiments that an increase in the net-synthesis of proteins is necessarily caused by the action of carcinogens since a mere study of incorporation of a labelled amino acid into proteins cannot solve this problem (Borsook, 1954). It cannot be excluded, for instance, that an inhibition of proteolytic processes claimed by some authors (Rondoni, 1955) is involved in this effect. These and some further aspects are being studied in detail in our laboratory to make clearer relations between protein metabolism and the action of chemical carcinogens.

SUMMARY

1. Suspensions of benzopyrene, dibenzanthracene, anthracene, phenanthrene, chrysene, cholesterol, and dehydrocholic acid in olive oil were administered in 1 mg. to 10^{-8} mg. doses to mice on the day preceding the intraperitoneal trans-

planatation of Ehrlich ascites carcinoma and the survival time in individual groups statistically evaluated.

2. The effect of olive oil suspensions of the above substances as well as that of methylcholanthrene, pyrene, deoxycholic acid, and sodium glycocholate in 1 mg. to 10^{-8} mg. doses on the incorporation of [^{35}S]methionine or [^{14}C]glycine into the proteins of Ehrlich ascites carcinoma and liver or spleen homogenate was tested.

3. Benzopyrene, dibenzanthracene, anthracene, and dehydrocholic acid in high doses reduces the tumour growth while in smallest doses they enhance this process. Chrysene stimulates the growth in all doses used while phenanthrene and cholesterol are without effect.

4. A specific effect on the incorporation of labelled amino acids into proteins was found with strong carcinogens, weak carcinogens and non-carcinogenic substances. Some doses of carcinogens stimulate this process while others inhibit it. One dose of maximal stimulating effect was found when using weak carcinogens while two such peaks were exhibited by strong carcinogens. Only an inhibitory effect was seen when highest doses of non-carcinogenic compounds were added, with the exception of anthracene in which a slight stimulating effect was caused by some of the highest doses.

5. Relations between the effect on protein synthesis and that on the tumour growth are discussed and the importance of protein synthesis affecting activity of carcinogenic substances is indicated.

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REFERENCES

BORSOOK, H.—(1954) in Greenberg's ' Chemical Pathways of Metabolism,' Volume II, New York (Academic Press Inc.).
CREECH, H. M.—(1940) *Amer. J. Cancer*, **39**, 149
EULER, H., AND SKARZYNSKI, B.—(1942) ' Biochemie der Tumoren,' Stuttgart (F. Enke Verlag).
HADDOW, A.—(1935) *Nature, Lond.*, **136**, 868.
HARTWELL, J. L.—(1951) ' Survey of Compounds Which Have been Tested for Carcinogenic Activity,' 2nd edn., Washington (U.S. Public Health Service).
HRADEC, J.—(1958*a*) *Nature, Lond.*, **182**, 52–(1958*b*) *Brit. J. Cancer*, **12**, 290 (1958*c*) Abstracts of papers, 7th International Cancer Congress, London.
Idem, DUŠEK, Z., TROJAN, K. AND PTÁČEK, M.—(1958) *Z. Krebsforsch.*, **62**, 387.
KLEIN, G. AND RÉVÉSZ, L.—(1953) *J. nat. Cancer Inst.*, **14**, 229.
KLEINENBERG, H. E., NEUFACH, S. A. AND SCHABAD, L. M.—(1941) *Cancer Res.*, **1**, 853.
MELCHIOR, J. B., AND HALIKIS, M. N.—(1952) *J. biol. Chem.*, **199**, 773.
Idem AND TARVER, H.—(1947) *Arch. Biochem.*, **12**, 309.
OWEN, S. E., WEISS, H. A. AND PRINCE, L. H.—(1939) *Amer. J. Cancer*, **35**, 424.
PATT, H. M. AND BLACKFORD, M. E.—(1954) *Cancer Res.*, **14**, 391.
QUASTEL, J. H.—(1958) Abstracts of Papers, 7th International Cancer Congress, London.
RAREI, B. AND GUMMEL, H.—(1938) *Z. Krebsforsch.*, **48**, 347.
RONDONI, P.—(1955) *Advanc. Cancer Res.*, **3**, 171.
SCHABAD, L. M.—(1945) *Cancer Res.*, **5**, 405.

STUDIES ON ISOLATED TUMOUR MITOCHONDRIA: BIOCHEMICAL PROPERTIES OF MITOCHONDRIA FROM HEPATOMAS WITH SPECIAL REFERENCE TO A TRANSPLANTED RAT HEPATOMA OF THE SOLID TYPE

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A BASIC policy in the study of neoplastic growth is to compare the metabolic characteristics of a tumour with those of the homologous normal tissue. In the long run this may lead to a better understanding of the neoplastic diseases and furnish some rational clue(s) for a chemotherapy of cancer.

The rat liver is a suitable tissue for study because of its relatively uniform composition, the ease of obtaining a sufficient quantity of tissue and the possibility of inducing experimental hepatomas with chemical agents. In the experiments to be reported in the present paper, mitochondria were isolated from a transplanted rat hepatoma and studied in respect to a number of important enzymic reactions. The original tumour (case No. BY 252) arose several years ago in a rat of our inbred colony as a result of feeding DAB*. The following favourable circumstances allow that a strict comparison can be made between the enzymic activities of the hepatoma and normal liver mitochondria. The intraperitoneally transplanted BY 252 rat hepatoma is composed of a uniform population of undifferentiated liver tumour cells classified as a carcinoma solidum. Since fibrous elements are virtually absent, the tumour shows the same softness as normal rat liver and homogenization of the two types of tissue can thus be performed in exactly the same way.

The levels of the free and DNP-activated ATPases, oxidative phosphorylation in the absence and presence of thyroxine and fatty acid oxidation of the hepatoma particles have been studied. The results of these experiments clearly showed that the hepatoma mitochondria were more labile *in vitro* than normal rat liver mitochondria.

Another transplanted rat hepatoma which has become a favourite test object in recent years is the Novikoff hepatoma (Novikoff, 1957). The latter tumour and the BY 252 hepatoma show approximately the same rate of growth and a similar histological structure; both have been induced by DAB. According to the Greenstein concept, undifferentiated tumours, as a class, tend to resemble each other metabolically more than they do their tissue of origin (Greenstein, 1953, 1956). The pertinent data on which this concept is based, apply not only to metabolic functions performed by organo-typical enzyme systems—such as those mediating the formation of urea in the liver—but also to enzymes which are

* Abbreviations are used as follows: DAB = *p*-dimethylaminoazobenzene, AAT = *o*-aminoazotoluene, ATP = adenosine triphosphate, DNP = 2,4 : dinitrophenol, DPN = diphosphopyridine nucleotide, NAA = nicotinamide, EDTA = ethylenediamine tetra-acetate, α-OC = α-oxycaproate.

among the basic functional equipment of practically every mammalian cell. The mitochondrial glutamic dehydrogenase and ATPase are considered to belong to the latter class of enzymes. Accordingly, it may be expected that the mitochondrial activities of the BY 252 hepatoma would bear more resemblance to those of the Novikoff hepatoma than to those of normal rat liver. The statement that "the glutamic dehydrogenase is the first enzyme known to be lacking in mitochondria of liver tumours" (Allard, de Lamirande and Cantero, 1957), based on the finding that no trace of the enzyme could be detected in isolated mitochondria or homogenates of the Novikoff hepatoma, might thus be warranted and have some genuine significance for the problem of neoplastic liver growth if a similar situation should exist in the case of the BY 252 hepatoma. The same consideration applies to the finding (Novikoff, 1957) that addition of DNP to homogenates of the Novikoff hepatoma does not lead to an enhanced dephosphorylation of ATP, which suggests that the tumour mitochondrial ATPases, in contrast to the corresponding enzymes in liver mitochondria, are in a non-latent state.

It will be shown, however, that a marked difference between the Novikoff and the BY 252 hepatomas does exist in that the particles of the latter tumour contain a high glutamic dehydrogenase activity and also latent ATPase activity. These results lead one to regard the two tumours as separate biological entities, each with its own distinct metabolic background. Accordingly, only the study of a number of tumours derived from the same tissue might reveal a common characteristic which distinguishes the particular tumour type from its homologous normal tissue.

Substantiation for a possible generalization of the concept that hepatoma mitochondria, as a class, are more labile than liver mitochondria has therefore been sought in the study of a number of different hepatomas, both primary and transplanted. For reasons of comparison a study of the mitochondria from a mammary ascites carcinoma of the mouse and from a sarcoma has been included.

MATERIALS AND METHODS

Tumours.—Liver tumours were induced over a period of 4 years by feeding DAB to approximately 250 female rats of the inbred strain R-Amsterdam which were kept on a diet of polished rice. Hepatomas of the mouse were obtained by administration of AAT to 125 CBA females fed *ad libitum* with the standard laboratory diet (Emmelot and van Vals, 1957*a*). Administration of the azo dyes was started when the animals were 1–2 months (rats) and 2–3 months (mice) of age. The rats received DAB (10 mg. in 0·2 ml. rape-oil) each day except at week-ends and the mice received AAT (1 mg.) once weekly at the start and increasing to twice, thrice weekly etc. until after 5 months AAT was administered each day except at week-ends. Rat hepatomas appeared after 4–12 months and mice hepatomas after 11–12 months. The administration was continued until the tumours became palpable. For certain experiments the animals were sacrificed before the tumours became palpable. In some experiments mouse liver together with small hepatoma nodules was used.

After cervical dislocation under ether anesthesia, the liver with adhering tumour(s) was quickly removed and hepatoma tissue was completely separated from the remaining liver. Representative parts of the tissues were fixed, stained

and subjected to microscopic examination. A detailed study was made of the histological structure of the rat tumours and livers. The expression " hepatoma " is used in the present investigation to denote malignant liver growth. The rat tumours were classified as solid (hepatocellular), adeno (cholangiocellular) or mixed types in a descriptive sense. Only the experiments with solid hepatomas are included in the present report. In addition a more differentiated solid type of tumour, *i.e.* the trabecular hepatoma, resembling liver tissue, was recognized. The histological structures, illustrating the case report of the BY 252 rat hepatoma (Fig. 1–6), are representative for the liver tumours observed in our rats.

The BY 252 rat hepatoma.—A hepatoma which arose in January 1956 in one of our rats (BY 252), following the ingestion of DAB during 5 months, was successfully transplanted by subcutaneous grafting. The primary tumour was of the mixed adeno-solid type (Fig. 1 and 2). In the slice prepared from the first transplant, adenohepatoma was found (Fig. 3). Until the 28th transplant generation the tumour was not subjected to further microscopic examination. The latter transplant was composed of solid hepatoma (Fig. 4). Subcutaneous transplantation led to a marked necrosis. The tumour was, therefore, subsequently transferred by the intraperitoneal route. At the moment the tumour is transplanted each ninth day. During this period the hepatoma grows rapidly as largely non-necrotic soft masses throughout the mesentery and peritoneum. When the tumour was allowed to grow longer, marked necrosis was found and the animals suddenly died (14 days). Some ascites formation was always observed; it was possible to transplant the tumour by using a small amount of ascites fluid—tumour masses always resulted. All transplants took in 100 per cent of the cases.

The intraperitoneally transplanted BY 252 rat hepatoma consists of undifferentiated hepatoma cells (Fig. 6) with a small rim of a more differentiated cell type which infiltrates the peritoneal fat (Fig. 5). Fibrous elements are virtually absent. The hepatoma is classified as a carcinoma solidum of the spindle cell type. Unless stated otherwise, only intraperitoneal transplants have been used in the present experiments.

Apart from the above tumours, another type of growth—resembling liver in colour and softness—was encountered in about 20 of the rats on the DAB diet. The fresh weight of these tissues varied from 5–40 grammes. Both histological and biochemical criteria allow the conclusion that these growths are of a non-malignant character. A detailed description of the histology (Fig. 7–12) of one case (rat BY 235, fed with DAB during 14 months) is given.

Rat BY 235

Macroscopy.—The right and middle liver lobes are without gross pathology. The left lobe is changed into a spherical " tumour " of 40 g. wet weight (the rest of the liver weighing about 5 g.). The cut surface has a speckled yellowish gray brown appearance. The " tumour " is partly surrounded by a thin layer of atrophic fatty liver tissue.

Microscopy.—As usual in these experiments, cellular and nuclear pleomorphism with fatty acid infiltration in right and middle lobes. No cirrhosis. Scattered small nodules of hyperplasia which may be basophilic (Fig. 11) or eosinophilic (Fig. 12). There are only occasional small foci of haemopoiesis. *Left lobe*: the " tumour " consists of a mixture of fatty cells and hyperplastic cells of basophilic type

(Fig. 7, 8 and 10). The picture is principally the same as in the small nodules in the right lobe. The hyperplastic cells are more pleomorphic than in the (altered) surrounding liver tissue and in the (altered) right lobe. It is impossible to state whether the fatty cells in the " tumour " are original liver cells or fatty infiltrated hyperplastic cells. In the " tumour " many foci of haemopoiesis (Fig. 9) are found ; far more than in the rest of the liver.

In ordinary malignant hepatomas haemopoiesis was found only as long as original liver tissue was included in the tumour. The whole swelling of the left lobe is histologically interpreted as to be of a non-malignant, not definitely tumourous, but hyperplastic nature. The name " hyperplastoma " may serve to distinguish this type of growth from hepatoma (the expression " hepatoma of low malignancy " is sometimes also used in the literature). These large, local hyperplastomas may be principally of the same nature as the small foci of hyperplasia that one finds so often scattered throughout the pre-neoplastic rat liver. No relation appeared to exist between these small foci and cirrhosis, although they are often combined. They should not be considered to be identical with the nodules in annular cirrhosis. In our opinion there exists a relation between these foci of hyperplasia and true (malignant) hepatoma, since isolated areas of small amounts of hepatoma cells could be observed in the hyperplastomas and hyperplastic foci. We think it possible that this form of hyperplastoma has its counterpart in the isolated cirrhotic hyperplastic " tumours " of the liver as seen in man (McBurney, Woolner and Wollaeger, 1950).

The transplanted mouse tumours (hepatomas and sarcoma UV 256), used in the present investigation, were well-established specimens induced and mantained in our laboratory for more than five years. The ascites tumour studied was the S3A mammary carcinoma originally obtained from Professor G. Klein (Stockholm).

Isolation of mitochondria.—The excised tissues were collected in small beakers placed in crushed ice and immediately cut into small pieces and homogenized at 0° C. in an all-glass Potter-Elvejhem type of apparatus with a loose-fitting pestle. Ascites cells were harvested from $F_1(B \times C_3H)$ mice inoculated 7–9 days before. The ascites fluid was removed and the cells were freed from erythrocytes by one suspension in physiological saline followed by low-speed centrifugation at 0° C. The cells were suspended in twice distilled water for 10–15 minutes and immediately homogenized after addition of sucrose and EDTA to give a final concentration of 0·25 and 0·001 M, respectively. The homogenization was carried out by using a rather tight-fitting pestle ; in some experiments the ascites cells were collected and immediately disrupted by sonic oscillation during 105 seconds (9 kc). One part of tissue and 9 parts of medium were used in the case of liver and liver tumours and 4–5 parts of medium with other tumours. In most experiments 0·25 M sucrose containing 0·001 M EDTA, pH 7·4, has been used as isolation medium for the mitochondria. As indicated in the text 0·25 M sucrose alone, or 0·25 M sucrose containing 0·01 M EDTA and 0·001 M NAA, has been used in certain experiments. In still other cases 0·23 M raffinose hydrate, 0·001 M EDTA and 0·085 per cent heparine in 6 per cent dextran served as medium (Birbeck and Reid, 1956). The dextran (MW 150·000) originally used was a gift from Glaxo Laboratories ; for later experiments dextran of MW 75·000 was purchased from Poviet Cy., Amsterdam, and included in the medium in a final concentration of 3 per cent.

Homogenization of the cells and finely-cut tissues was carried out by gently passing the tube, 6 times along the rotating pestle (1400 r.p.m.), which had to be carefully centered, avoiding too much manual pressure. The homogenization lasted for about 25–35 seconds ; any tissue left intact after this treatment was discarded.

The homogenates were centrifuged for 10 minutes at 700 × g and 0° C. Only the middle part of the tube content was used for the subsequent isolation of the mitochondria ; the surface layer, which mainly consisted of fat droplets, was carefully removed and a rather ample layer above the nuclear pellet was discarded. This procedure appeared to be important in order to obtain tumour mitochondria with a relatively low ATPase activity. The nuclear pellet was not washed. Mitochondria were spun down at 5000 × g during 10 minutes (the low gravitational field was chosen in order to minimize the risk of microsomal contamination), the " fluffy layer was removed and the pellet was resuspended and washed once (ATPase assay) or twice (respiration study). The fresh mitochondria were always immediately used. In a number of experiments the particles were disrupted either by freeze-thawing (— 15° C. → + 5° C ; 3 or 6 times) or by sonic oscillation with 9 kc at 50 Watt in the Raytheon oscillator during 10 minutes, unless otherwise stated.

Measurements.—For the ATPase assay 0·1 ml. mitochondrial suspension and 0·1 ml. 0·1 M ATP was added to 1·6 ml. containing 0·1 M KCl, 0·05 M TRIS and 0·005 M $MgSO_4$, pH 7·2; DNP present in a final concentration of 10^{-4} M. Incubation unless otherwise stated at 27° C. Reaction stopped by adding 0·1 ml. 50 per cent trichloroacetic acid. Inorganic phosphate measured according to Fiske and Subbarow (1929).

The experiments in which the oxidative properties of the primary rat and mouse hepatoma mitochondria were studied (Tables X and XI) have been conducted as described earlier (Emmelot and Bos, 1955, 1957*a*, at pH 7·4 in phosphate buffer). The other experiments (Tables VIII, XI, XII and XIII) have been carried out in histidine buffer pH 7·0 (Emmelot, 1957*a*). The experiments with thyroxine were always performed at pH 7·4 either in phosphate or in histidine buffer. 0·1 ml. of DL-thyroxine (obtained from Hofmann and la Roche) in 0·004 M NaOH was added to the respirometer flask to give a final concentration of $7{\cdot}5 \times 10^{-5}$ M. Total fluid volume was always 1·6 ml. Hexanoate and octanoate 2 μmoles, pyruvate 24 μmoles, glutamate 56 μmoles, β-hydroxybutyrate 12 μmoles. The O_2-consumptions have always been corrected for blanks obtained in the absence of the particular substrates.

It should be noted that in those experiments of Table X and Table XI in which α-oxycaproate (0·003 M) was added as a primer of fatty acid oxidation (Emmelot and Bos, 1955), octanoate is completely converted to acetoacetate when 134 μl of O_2 are consumed. In the presence of L-malate (0·0003 M), however, oxidation via the citric acid cycle becomes possible. In a number of experiments DL-β-hydroxybutyrate was incubated in the absence of a primer since the oxidation of the D(−)isomer to acetoacetate, in contrast to that of the L(+)isomer, is independent of ATP and coenzyme A. 1 Mg. hexokinase (grade III, Sigma) containing 153 K.M. units at 25° C was added as indicated. The serum albumin used was of human origin and prepared in the Central Laboratory of the Netherlands Red Cross (Amsterdam). It was thoroughly dialyzed before use to remove oxidizable substrates.

RESULTS

ATP-Dephosphorylation

Freshly-prepared intact mitochondria of rat liver contain a so-called latent adenosine triphosphatase. ATPase activity can be manifested in these particles either by 2 : 4-dinitrophenol (DNP-activated ATPase) or by physical and chemical agents which cause damage to the integrated structure of the mitochondria (Mg^{2+}-activated ATPase) (Kielley and Kielley, 1951; Potter, Siekevitz and Simonson, 1953; Siekevitz, Löw, Ernster and Lindberg, 1958). The DNP-activated ATPase is generally considered (Hunter, 1956) to represent a reversal and diversion of the reactions which are responsible for the synthesis of ATP during the process of oxidative phosphorylation, whereas the Mg^{2+}-activated ATPase probably represents some mal-function resulting from a disorganization of the otherwise integrated enzymic machinery of the latter process. It has been shown that progressive ageing of isolated liver mitochondria leads to a labilization of the integrated functions as shown by the loss of DPN, a gradual decrease in the efficiency of oxidative phosphorylation and an increase in free (Mg^{2+}-activated) ATPase. Concomitantly, the DNP-activated ATPase decreases so that eventually a situation may be reached in which DNP no longer activates the ATPase in the presence of Mg^{2+} (Potter *et al.*, 1953; Siekevitz *et al.*, 1958). Hence the presence of free ATPase activity and the level of the DNP-activated ATPase relative to the former activity, may provide some information about the degree of intactness of the biochemical features of isolated mitochondria.

The BY 252 hepatoma, S_3A ascites carcinoma and UV 256 sarcoma

Table I illustrates the dephosphorylation of ATP by fresh mitochondria isolated from the BY 252 rat hepatoma in 0·25 M sucrose containing 0·001 M EDTA and incubated at 27° and 37° C in the absence and presence of Mg^{2+} and/or DNP (10^{-4} M). Dephosphorylation is recorded as the micrograms phosphor liberated from ATP. On account of the non-proportionality of enzyme activity to enzyme concentration (Potter *et al.*, 1953; Emmelot, Bos and Brombacher, 1956) no attempt has been made to express the present data on a uniform weight basis. Since some variation was observed with individual preparations each experimental condition has been illustrated by a number of results.

ATP-dephosphorylation in the absence of Mg^{2+} and DNP varied from almost zero to moderate values (experiments 2*c*, 3*e*, and 4*e*, respectively). In the presence of DNP and the absence of Mg^{2+} (experiments 2*d* and 4*f*), and *vice versa* (experiments 2*a* and 4*a*), enhanced activity was noted. The fresh hepatoma mitochondria thus contained a Mg^{2+}- and a DNP-activated ATPase at 27° C. The Mg^{2+}-activated ATPase of the fresh tumour particles was more active and the DNP-activated enzyme less active than the corresponding enzymes in the mitochondria of normal liver of rats of the same inbred strain as that from which the hepatoma was derived (Table II).

The ATP-dephosphorylation by the hepatoma mitochondria at 27° C in the presence of DNP and Mg^{2+} was always higher than that in the presence of Mg^{2+} alone (experiments 1*b*, 2*b*, 3*b*, 4*b*, 5*b*). Thus, in the presence of free ATPases the DNP-activated ATPase could be manifested. However, it is evident that the effect of DNP is smaller at higher levels of the free (Mg^{2+}-activated) ATPase. The

latter is more clearly illustrated by the results of similar experiments carried out at 37° C.

The Mg^{2+}-activated ATPase of the hepatoma mitochondria was always more active at 37° C (experiments 3*c*, 4*c* and 5*b*) than at 27° C. Apart from the

TABLE I.—*Effect of DNP and/or Mg^{2+} on ATP-dephosphorylation by fresh mitochondria from the BY 252 rat hepatoma at 27° and 37° C.*

Isolation mitochondria in 0·25 M sucrose containing 0·001 M EDTA.

Experiment No.	Amount of mitochondria (mg. N/flask)	Temperature of incubation (°C.)	Mg^{2+}	DNP	µg. phosphor released after minutes		
					5	10	20
1 *a*	0·04	27	+	−	8	12	16
b		27	+	+	12	40	65
2 *a*	0·04	27	+	−	2	14	34
b		27	+	+	18	27	48
c		27	−	−	0	2	6
d		27	−	+	9	12	16
3 *a*	0·07	27	+	−	6	17	34
b		27	+	+	30	42	51
c		37	+	−	11	31	58
d		37	+	+	30	50	68
e		37	−	−	0	11	20
4 *a*	0·07	27	+	−	15	25	63
b		27	+	+	28	51	92
c		37	+	−	22	46	96
d		37	+	+	33	52	94
e		27	−	−	7	17	25
f		27	−	+	32	42	67
5 *a*	0·05	27	+	−	8	25	36
b		27	+	+	33	62	70
c		37	+	−	26	49	75
d		37	+	+	36	58	80

TABLE II.—*Effect of DNP on ATP-dephosphorylation by fresh rat liver mitochondria at 27° and 37° C.*

Isolation of mitochondria in 0·25 M sucrose containing 0·001 M EDTA.

Amount of mitochondria (mg. N/flask)	Temperature of incubation (°C.)	Mg^{2+}	DNP	µg. phosphor released after minutes		
				5	10	20
0·04	27	−	−	0	0	2
	27	−	+	25	61	95
	27	+	−	0	5	15
	27	+	+	40	65	118
	37	+	−	8	14	31
	37	+	+	50	105	156
0·06	27	+	−	0	0	8
	27	+	+	60	89	123
0·05	37	+	−	18	28	36
	37	+	+	67	102	155

effect of the temperature on the reaction rate, the higher activity might also have been due to an increased labilization of the particles, which developed the Mg^{2+}-activated ATPase more or less completely and thus depressed the reaction sequence of the DNP-activated ATPase. The absence of any marked effect of DNP on the ATP dephosphorylation in the presence of Mg^{2+} at 37° C observed in two experiments (experiments 4*d* and 5*d*) during the whole period of incubation shows that the latter situation may indeed be realized. In another experiment (3*c*) the particles were somewhat more resistant to the higher temperature so that the Mg^{2+}-activated ATPase did not develop completely and DNP (experiment 3*d*) still showed some effect.

As illustrated in Table III mitochondria prepared from the S3A ascites mammary carcinoma behaved similarly. A marked effect of DNP on ATP-dephosphory-

TABLE III.—*Effect of DNP on ATP-dephosphorylation by mitochondria and homogenates prepared from the S3A ascites carcinoma and by mitochondria from the UV 256 sarcoma.*

Isolation mitochondria in 0·25 M sucrose containing 0·001 M EDTA. Incubation in the presence of Mg^{2+}.

Source of enzyme	Temperature of incubation (°C.)	DNP	µg. phosphor released after minutes		
			5	10	20
S3A ascites mitochondria (0·03 mg. N/flask)	27	−	8	15	30
	27	+	20	35	61
	37	−	14	36	90
	37	+	42	62	96
S3A ascites homogenate (4·7 mg. dry weight/flask)	27	−	10	16	31
	27	+	30	37	53
Ditto (4·0 mg. dry weight/flask)	37	−	10	19	45
	37	+	55	80	85
UV 256-sarcoma mitochondria (0·12 mg. N/flask)	27	−	..	21	52
	27	+	..	80	148
(0·10 mg. N/flask)	27	−	..	35	58
	27	+	..	64	103

lation in homogenates of ascites cells was also observed at 27° and 37° C. Microscopic analysis has shown that in these homogenates approximately 10 per cent intact cells were present. The effect of DNP on the ATP-dephosphorylation of the homogenates was not due to a stimulation of the ATPase of the remaining intact cells since in parallel experiments it was found that DNP exerted a negligible effect on the ATP-dephosphorylation of freshly harvested ascites cells at 27 and 37° C. The ATPase of sarcoma mitochondria (tumour UV 256 of the mouse) may also be activated by DNP at 27° C. In the two experiments illustrated in Table III the latter effect was very evident; in a number of other experiments a less distinct effect was observed.

In the case of normal rat liver mitochondria the higher incubation temperature (37° C) evoked also the Mg^{2+}-activated ATPase but the level of this activity at 37° C was, in contrast to the situation in the hepatoma mitochondria, only a small fraction of that reached when DNP was present in addition (Table II).

The ATP-dephosphorylation by disintegrated hepatoma and liver mitochondria is illustrated in Table IV. The particles were either freeze-thawed or sonicated to ensure complete structural disorganization and, thus, to uncover the potential ATPase content. ATP-dephosphorylation by the disrupted liver and hepatoma particles in the presence of Mg^{2+} was similar to that observed with untreated particles in the presence of DNP and Mg^{2+}. Addition of DNP to the disrupted mitochondria had little effect in the case of liver and no effect at all in the case of hepatoma (Mg^{2+} present).

TABLE IV.—*Effect of DNP on ATP-dephosphorylation by fresh and disrupted hepatoma and liver mitochondria at 27° C*

Mitochondria isolated in 0·25 M sucrose containing 0·001 M EDTA. Incubation in the presence of Mg^{2+}.

Mitochondria from (mg. N/flask)	Condition	DNP	µg. phosphor released after minutes 5	10
BY 252 rat hepatoma (0·07)	Fresh	−	9	15
		+	18	40
	Sonicated	−	20	33
		+	18	40
	Freeze-thawed	−	20	35
		+	24	35
(0·05)	Fresh	−	4	10
		+	12	26
	Sonicated	−	11	24
		+	13	25
Rat liver (0·07)	Fresh	−	0	7
		+	59	97
	Sonicated	−	55	99
		+	63	113

The data presented in Tables I to IV allow the conclusion that freshly prepared mitochondria of the BY 252 rat hepatoma are more labile than rat liver mitochondria and especially so at a temperature of 37° C. The enhanced lability of the hepatoma particles leads to the spontaneous development of the Mg^{2+}-activated ATPase and to a decrease in activity of the DNP-activated enzyme. However, as long as the Mg^{2+}-activated enzyme is not completely manifest, a distinct effect of DNP can be observed. It appears further that the DNP-activated ATPase of the hepatoma particles, both in the absence and in the presence of Mg^{2+}, is smaller than that of liver mitochondria. This applies also to the potential ATPase-content, as manifested in disrupted mitochondria by Mg^{2+}.

The data of Table I show that separate mitochondrial preparations of the BY 252 rat hepatoma may vary somewhat in the degree of lability, This variability was also observed in the efficiency of oxidative phosphorylation and fatty acid oxidation of the particles (compare below). This phenomenon is probably connected with the physiological state of the tumour since "young" tissue (less than 9 days after transplantation) gave the best results.

Primary rat hepatomas, rat liver hyperplastomas, primary and transplanted mouse hepatomas.—Mitochondria from primary DAB-induced hepatomas of the solid type and from certain other tumours (Emmelot, Bos and Brombacher,

1956; Emmelot and Bos, 1957*c*; Potter *et al.*, 1953) have earlier been found to possess an appreciable free ATPase activity which, except in the case of spontaneous mouse hepatomas, could not be stimulated by DNP. A lack of response of the ATP-dephosphorylation to DNP has been observed in homogenates of primary rat hepatomas and other tumours (Novikoff, 1957). Free ATPase activity in particles of primary rat hepatomas (15 minutes at 12·000 × g., "fluffy layer" not removed) has also been noted by other investigators (Reid and O'Neil, 1956). The latter results are, however, difficult to interpret since microsomal material (which in tumours may possess appreciable ATPase activity) of the "fluffy layer" might have contributed to the observed activities.

After carrying out the isolation as described under Materials and Methods we have been able in the last two years to prepare mitochondria from primary rat hepatomas (0·25 M sucrose containing 0·00 1M EDTA) which show an enhanced ATP-dephosphorylation on addition of DNP at 27° C. Some of these results, including those obtained with mitochondria from mouse hepatomas, are listed in Table V. In these experiments the level of the free ATPase and the effect of DNP was of the same order as that observed with the BY 252 hepatoma mito-

TABLE V.—*Effect of DNP on ATP-dephosphorylation by fresh mitochondria from primary rat and mouse hepatomas, transplanted mouse hepatomas and rat hyperplastomas*

Mitochondria isolated in 0·25 M sucrose containing 0·001 M EDTA.
Incubation in the presence of Mg^{2+}.

Mitochondria from	Amount of mitochondria (mg. N/flask)	DNP	μg. phosphor released after minutes 5	10	20
Primary rat hepatomas, solid type	0·07	−	8	18	33
		+	9	20	64
	0·05	−	7	13	23
		+	14	27	49
	0·06†	−	6	12	35
		+	26	42	55
	0·06	−	6	10	35
		+	12	25	45
Primary mouse hepatomas	0·04	−	0	5	14
		+	5	26	52
		+*	30	42	58
	0·30	−	46	90	142
	0·27	−	26	65	87
Transplanted mouse hepatomas:					
T 28012	0·04	−	6	13	25
		+	12	26	51
T 26473	0·04	−	8	17	34
		+	16	35	59
	0·25	−	41	68	114
Hyperplastoma, rat	0·05	−	5	8	15
		+	11	24	45
Hyperplastoma/solid hepatoma	0·06	−	3	13	32
		+	37	67	102
(With some cirrhotic liver rests)	0·03	−	0	6	18
		+	25	36	62

* γ-globulin added (compare Emmelot and Bos, 1957*c*).
† BY 400, compare for liver Table VI.

chondria. Sometimes the effect of DNP developed sluggishly (compare the first experiment of Table V). Disrupted mitochondria of primary rat hepatomas did not show a higher ATP-dephosphorylation (Mg^{2+} present) than that observed with fresh particles in the presence of DNP and Mg^{2+}.

The mitochondria of some primary AAT-induced mouse hepatomas and of all " hyperplastomas ", found in rat livers as a result of DAB feeding, showed a more " normal " behaviour in the presence of DNP (Table V). The oxidative properties of the hyperplastoma mitochondria have also been found to resemble more those of normal liver mitochondria than of malignant hepatoma mitochondria (Table X). The non-tumourous character of the hyperplastomas is further illustrated by the following biochemical criteria. The glucose-6-phosphate dehydrogenase, the reduced triphosphopyridine nucleotide diaphorase and -cytochrome *c* reductase contents of the combined microsomal soluble fractions of the hyperplastomas have in four experiments been found to be similar to those of liver of control-rats fed with polished rice for prolonged periods and of liver from which hepatomas (13 experiments) had been removed. The levels of these three enzymes amounted to, respectively, 120, 50 and 50 per cent of those of livers of rats fed the standard diet. In solid, adeno or mixed hepatomas (10 experiments) the glucose-6-phosphate dehydrogenase had increased to 200–300 per cent and the diaphorase and cytochrome *c* reductase dropped to 10–20 per cent of the normal level. These latter data may be compared with those reported for the Novikoff hepatoma, which show that the glucose-6-phosphate dehydrogenase is increased for 500 per cent (Weber and Cantero, 1957) whereas the TPNH-cytochrome *c* reductase was absent (Reynafarje and Potter, 1957).

Pre-neoplastic rat liver.—Since the DAB-fed rats had been placed on a protein- and vitamin-deficient diet, it was investigated next whether this might have had any effect on the level of the mitochondrial ATPases. Liver mitochondria of rats receiving the standard laboratory diet and of rats fed polished rice showed the same low ATPase activity and the effect of DNP was also similar in the two cases, both at pH 7·2 and 8·5 (Table VI). At pH 8·5, the free ATPase activity was higher and the DNP-activated ATPase lower than the corresponding activities at pH 7·2. ATP-dephosphorylation by fresh liver mitochondria in the presence of Mg^{2+} and DNP at pH 7·2 was equal to that obtained with sonically disrupted particles in the presence of Mg^{2+}. However, at pH 8·5 the ATP-dephosphorylation under the latter conditions was distinctly higher than that of fresh particles incubated in the presence of Mg^{2+} and DNP.

Mitochondria from liver tissue dissected free from adhering hepatomas or from livers in which the hepatomas had not yet appeared behaved more or less like normal liver mitochondria; the free ATPase was sometimes enhanced (compare Clerici and Cudkowicz, 1956). These livers showed one or more of the following pathological changes: cirrhosis, fatty infiltration, hyperplastic and regenerative foci, cell and nuclear polymorphy and cholangiofibrosis. In some mitochondrial preparations of livers in which the latter changes were very marked, DNP was less active (last experiments of Table VI and Fig. 13).

Abolishment of Pasteur-effect in hepatoma slices by DNP.—An effect of DNP on mitochondrial processes could also be shown with intact tumour cells (Emmelot and Bos, unpublished). The anaerobic glycolysis of the tumours (at 37° C, Table VII) was higher than the aerobic glycolysis, but in the presence of DNP the aerobic lactate production was equal to the anaerobic one. In view of the inter-

TABLE VI.—*Effect of DNP on ATP-dephosphorylation by fresh and disrupted liver mitochondria of rats fed the standard laboratory diet, polished rice and polished rice plus DAB*

Incubation during 10 minutes at 27° C at pH 7·2 and 8·5, in the presence of Mg^{2+}. Isolation of mitochondria in 0·25 M sucrose containing 10^{-3} M EDTA ($s + v$) or in raffinose-dextran-heparine-EDTA ($r + d$).

The rats received polished rice during 6–7 months and polished rice plus DAB during 5–7 months (BY 387 and BY 400 : 7 months ; BY 384 A : 6 months ; BY 436 : 5 months).

Liver Mitochondria from	Amount of mitochondria (mg. N/flask)	pH	µmoles phosphate released by mitochondria			
			Fresh		Sonicated	
			− DNP	+ DNP	− DNP	+ DNP
Rat fed standard (s + v)	0·14	7·2	0·3	3·8	3·8	4·6
		8·5	1·0	3·3	4·6	5·6
Rat fed polished rice (s + v)	0·10	7·2	0·4	3·3	3·9	4·3
		8·5	0·9	2·9	5·0	5·9
Rat fed standard (r + d)	0·09	7·2	0·2	3·0	2·9	3·7
		8·5	0·6	1·6	4·0	5·5
Rat fed polished rice (r + d)	0·06	7·2	0·2	2·0	2·3	3·4
		8·5	0·6	1·4	3·4	3·8
Rats fed polished rice plus DAB, hepatomas removed :						
BY 387, cirrhosis light degree (r + d)	0·08	7·2	0·4	2·6	..	..
		8·5	1·0	2·1	..	..
BY 384 A, steatosis cell polymorphy, hyperplastic areas (r + d),	0·06	7·2	0·2	1·6	2·3	2·7
		8·5	0·5	1·2	3·6	3·6
BY 400, severe steatosis (s + v)	0·05	7·2	0·1	1·1	..	..
			(0·2)*	(1·7)*		
BY 436, whole liver affected by severe annular cirrhosis with cell polymorphy and marked cholangiofibrosis ; no hepatoma present† (s + v)	0·03	7·2	0·4	0·9	1·2	1·4
			(0·9)*	(1·8)*	(1·8)*	(2·1)*

* After 20 minutes.
† Compare Fig. 13.

TABLE VII.—*The stimulatory effect of DNP on the aerobic glycolysis of rat hepatomas of the solid type*

Each flask contained 100 mg. of wet weight slices suspended in 1·6 ml. Krebs-Ringer bicarbonate buffer. Incubation was carried out during 45 and 60 minutes, respectively, at 37° C. Aerobic conditions : 95 per cent O_2 + 5 per cent CO_2 ; anaerobic conditions : 95 per cent N_2 + 5 per cent CO_2. 10^{-4} M DNP added as indicated. Lactic acid determined according to Barker and Summerson (1941).

Tumour	Condition	DNP	Lactate produced (µmoles)
Primary hepatoma	Anaerobic	−	10·1
	Anaerobic	+	9·2
	Aerobic	−	5·0
	Aerobic	+	8·9
Transplanted BY 252 hepatoma	Anaerobic	−	11·0
	Anaerobic	+	14·2
	Aerobic	−	8·4
	Aerobic	+	19·0

pretation of the Pasteur effect in terms of a competition between respiration and glycolysis for inorganic phosphate and adenine nucleotides (Johnson, 1941; Lynen 1941, 1958; Racker, 1956) it follows from the latter results that DNP uncoupled the oxidative phosphorylations in the respiratory chain of the tumour mitochondria *in situ*. Moreover, if the interpretation of the Pasteur effect is correct, its occurrence in slices of the hepatomas must mean that the ATPases of the mitochondria of intact tumour cells are at least in a partially, if not a completely, latent state at 37° C. The non-latency of the ATPase activity of the isolated BY 252 hepatoma mitochondria at 37° C is thus due to the *in vitro* conditions.

Comment.—The present results demonstrate that DNP may activate the ATPase of isolated tumour, including hepatoma, mitochondria. The reported absence of such an effect in homogenates of the Novikoff and primary rat hepatomas (Novikoff, 1957) stands in marked contrast to our results. Novikoff's suggestion that the lack of response to DNP might be a common property of undifferentiated tumours is therefore not warranted. The opposite is true, activation of the latent ATPase by DNP appears to be common property of mitochondria from both sarcomas and carcinomas, provided that the particles are isolated in a reasonably intact and pure condition. A drastic homogenization, poor isolation technique

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EXPLANATION OF PLATES

(All slides 6 mμ, fixation in Susa, stained with haematoxylin azophloxin.)

FIG. 1–6.—*The BY 252 rat hepatoma* (all magnifications × 550).

FIG. 1, 2.—Primary tumour (malignant hepatoma). Partly adenomatous mucous producing pattern with cellular and nuclear pleomorphism (Fig. 1); partly solid, trabecular, rather regular pattern resembling liver tissue (Fig. 2).

FIG. 3.—First subcutaneous transplant. The slide consists of adenocarcinoma (adenohepatoma) with marked plecmorphism and slight mucus production.

FIG. 4.—Subcutaneous transplant, 28th passage. Solid non-trabecular growing tumour with pleomorphism, marked necrosis and inflammation.

FIG. 5, 6.—Intraperitoneal transplant, 34th passage. The outer cells show more or less an epithelial pattern, infiltrating the peritoneal fat (Fig. 5). The rest (greatest part) of the tumour is composed of fusiform cells (sarcoid) with suggesting of interweaving bundles (Fig. 6). All further transplants were of similar structure (carcinoma solidum, spindle-cell type).

FIG. 7–12.—*Rat BY 235 liver* (5 g.) *and hyperplastoma* (40 g.).

FIG. 7.—"Hyperplastoma" in left lobe. Upper part of figure shows capsule of liver rest with fatty infiltration. The hyperplastoma contains "liver" cells (or newly formed hyperplastic cells) with fatty infiltration. The smaller, darker cells are of hyperplastic basophilic type. × 125.

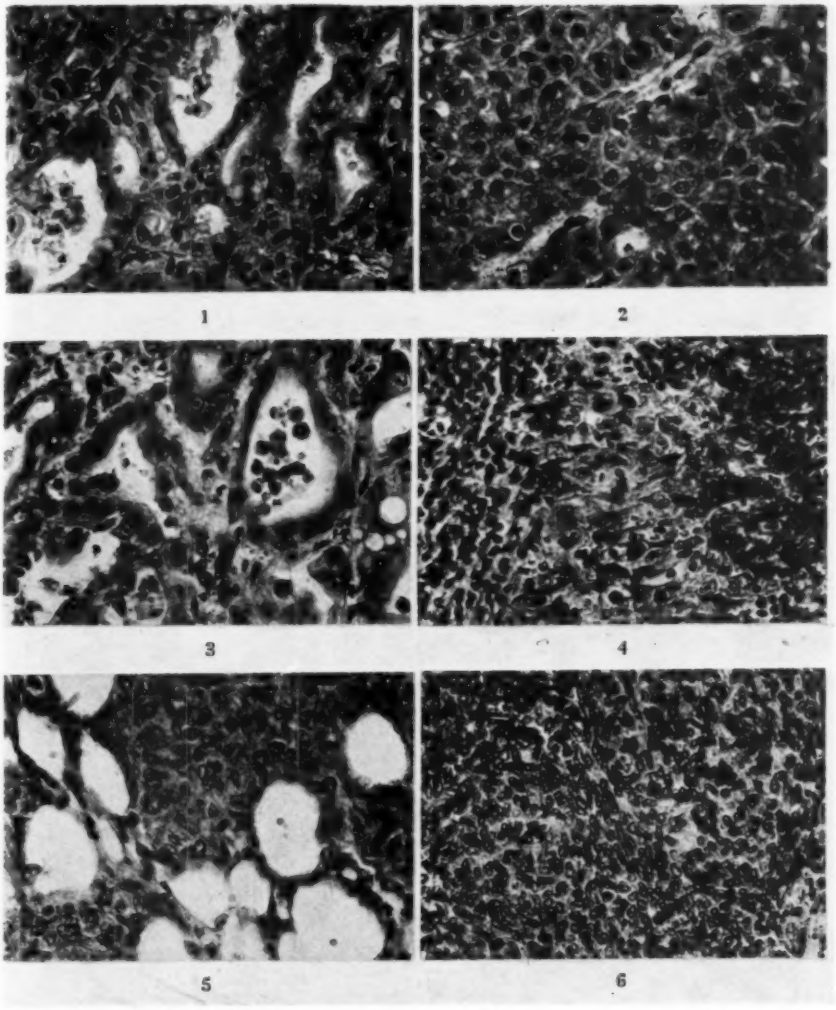
FIG. 8, 9.—Borderline of same hyperplastoma as illustrated in Fig. 7. Rest of fatty liver is seen at top (Fig. 8, × 175). Small dark cells in hyperplastoma are foci of haemopoiesis, which are seen in detail in Fig. 9. × 500.

FIG. 10.—Detail of hyperplastoma. Trabecular arrangement of newly formed cells with patent capillaries lined by endothelium (without typical Kupfer-cells). × 500.

FIG. 11.—Basophilic focus of hyperplasia in right lobe. Fatty infiltration. No cirrhosis. × 30. Nuclear pleomorphism is evident at higher magnification.

FIG. 12.—Eosinophilic nodule of hyperplasia in fatty right lobe. × 30.

FIG. 13.—*Rat BY 436 liver.*
Nodular cirrhosis of entire liver (9 g.). Scale in centimetres. Microscopic: annular cirrhosis with pleomorphic "hyperplasia" of gall ducts (cholangiofibrosis).



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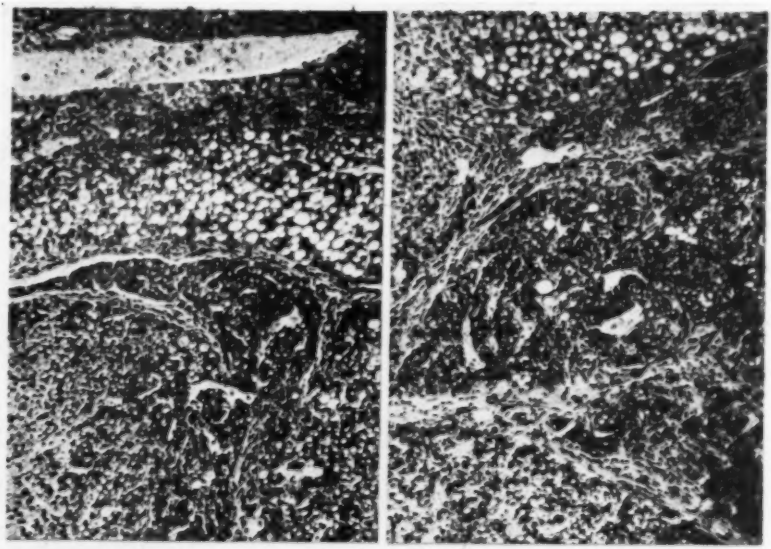
Emmelot, Bos, Brombacher and Hampe.



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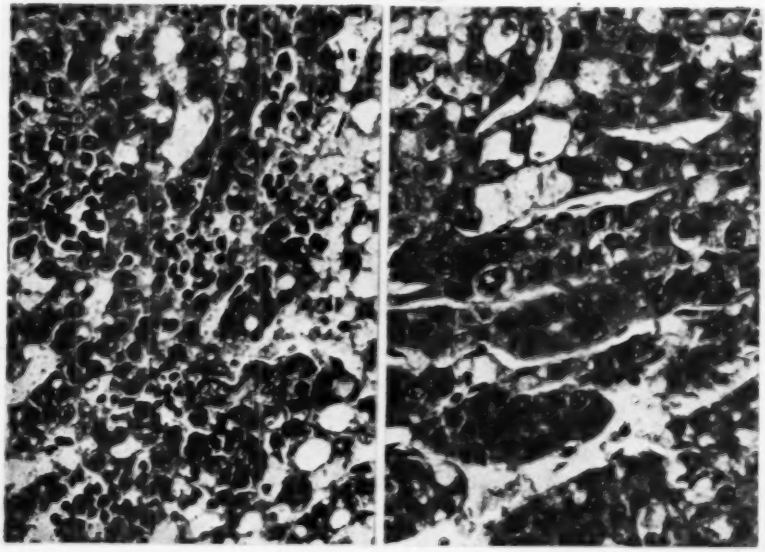

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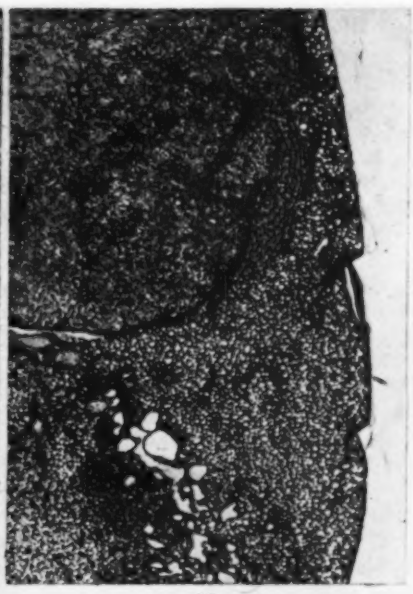

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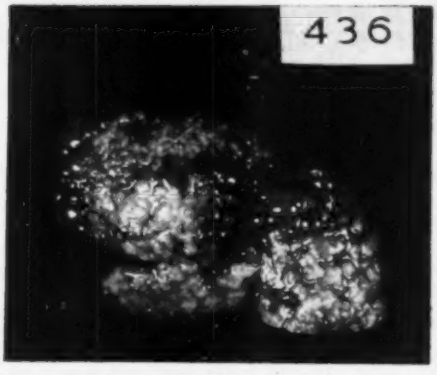

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and the use of mitochondria from "old" tumour transplants (necrosis) may, however, lead to negative results.

Oxidation and Phosphorylation

The BY 252 rat hepatoma: DPN-linked oxidations

Tissue slices (150 mg. wet weight) of the 28th subcutaneous transplant series of the BY 252 rat hepatoma were incubated during 120 minutes with 0·25 μmol DL-glutamate-1-C^{14} and approximately 25 per cent of the isotope was recovered in the respiratory carbon dioxide. Conclusive evidence for the presence of the glutamic dehydrogenase came from experiments conducted with the 33rd- and following intraperitoneal transplant generations, in which it was found that the mitochondria from these tumours, isolated in 0·25 M sucrose containing 0·001 M EDTA, were capable of oxidizing glutamate.

The oxidation of glutamate in the presence of DPN and NAA* was always high (Table VIII). A phosphorus to oxygen ratio of 1·0–1·8 in the absence and of 2·2–2·5 in the presence of the "high-energy" phosphate trapping system glucose-hexokinase was obtained in various experiments. The added hexokinase may thus compete successfully with the free ATPases of the particles for the ATP formed during oxidative phosphorylation. The variation in P : O ratio from 1·0–1·8 observed in the various experiments suggests that the free ATPase (or the degree of coupling) in some preparations was higher than in others. A similar conclusion was reached above.

When no DPN was added to the flasks the oxidation of glutamate by particles, isolated either in the presence or in the absence of 0·001 M NAA, but always incubated in the presence of NAA, was only 20–25 per cent of that recorded with DPN present.

Pyruvate oxidation.—The effect of DPN addition on the oxidation of pyruvate by mitochondria isolated from the BY 252 hepatoma in 0·25 M sucrose containing 0·001 M EDTA and 0·001 M NAA, was also studied (Table VIII). It appeared that the oxygen consumption in the absence of DPN was approximately 55 per cent of that found in the presence of DPN. It follows from these results that relatively more DPN was available to the enzymes oxidizing pyruvate than to those oxidizing glutamate. This may be connected with the site of the particular apoenzymes since the pyruvic dehydrogenase is known to be bound to the mitochondrial membranes whereas the glutamic dehydrogenase is located in the soluble part of the mitochondria (Hogeboom and Schneider, 1953). The poor O_2-consumption of the hepatoma particles in the absence of added DPN might have been due to the fact that the mitochondrial DPN-complement was already low *in situ* or that the DPN was lost from the mitochondria during their isolation and/or incubation. The loss could occur by the release of DPN (less intact membranes, less binding to proteins) or to the splitting of the coenzyme by an active DPNase. The latter was evidently not the case since the DPNase inhibitor NAA (added to the isolation or incubation medium) did not affect the rate of the oxidations. EDTA, on the other hand, which is known to protect against the loss of DPN from mitochondria (Slater and Cleland, 1953; Ernster, 1956; Emmelot and Bos,

* As a precaution NAA was added in all experiments to the respirometer flasks to prevent the enzymic splitting of DPN; in a number of experiments NAA was omitted but no change in the oxygen consumption was observed, indicating that the DPNase was not very active.

TABLE VIII.—*Oxidation and phosphorylation by mitochondria from the transplanted BY 252 rat hepatoma*

Mitochondria isolated in 0·25 M sucrose containing 10^{-3} M EDTA, or 10^{-2} M EDTA (if marked with †), and 10^{-3} M NAA (if marked with *). Incubation as described (Emmelot, 1957*a*). NAA (10^{-3} M) and DPN (10^{-3} M) present in each flask which contained glutamate or pyruvate unless otherwise stated in parentheses. EDTA added to the incubation flasks as indicated in a final concentration of 5×10^{-3} M EDTA. Hex-G stands for hexokinase and glucose. Pyruvate oxidation in the presence of L-malate (0·0003 M).

Amount of mitochondria (mg. N/flask)	Time of incubation (min.)	Substrate	Additions	ΔO (μatoms)	ΔP (μmoles)	P : O
0·52	15	Glutamate	..	6·5	10·1	1·5
			(No NAA)	6·3	9·9	1·5
0·56	20	Glutamate	..	6·6	11·9	1·8
			Hex-G	5·8	14·5	2·5
0·61	15	Succinate	..	3·6	2·9	0·8
0·94	15	Glutamate	..	6·6	7·9	1·2
		Succinate	..	4·4	6·5	1·4
0·47	15	Glutamate	Hex-G	4·7	10·8	2·2
	20	Succinate	..	3·4	3·4	1·0
0·67	15	Glutamate	(No DPN)	1·1	2·2	2·0
			(No DPN)*	1·2	2·5	2·1
			.. *	4·4	7·9	1·8
0·60	30	Pyruvate	(No DPN)*	2·4	..	..
	60		(No DPN)*	4·2	..	..
	30		.. *	4·2	.	..
	60		.. *	7·6	..	..
	60		EDTA (No DPN)*	7·8	..	..
0·80	60	Pyruvate	(No DPN)†, *	7·8	..	..
			.. †, *	7·9	..	..
0·50	20	Glutamate	(No DPN)*	2·6	3·2	1·2
			.. *	9·4	14·1	1·5
			EDTA (No DPN)*	5·3	10·6	2·0
0·47	20	Glutamate	(No DPN)†, *	4·8	..	..
			.. †, *	6·7	..	..
Rat liver—						
0·68	15	Glutamate	(No DPN)	6·5	16·3	2·5
		Glutamate	..	7·0	15·5	2·2
		Succinate	..	7·0	11·9	1·7

1957*a*) was found to raise the O_2 consumption of the hepatoma particles both with glutamate and pyruvate as the substrates, when it was included in the isolation medium in a concentration of 0·01 instead of 0·001 M or in the final incubation medium (0·005 M) in which mitochondria prepared in 0·001 M EDTA were present.* The latter finding indicated that in the absence of sufficient EDTA the release of DPN from the mitochondria occurred very early during the incubation of the particles at 27° C.

* Since 0·3 ml. of this suspension was added to the flasks to give a final volume of 1·6 ml., 0·00019 M EDTA was finally present. This concentration was apparently too low to counteract the release of DPN from the mitochondria (binding of Ca^{2+} ?), although it had been active in this respect with mitochondria from spontaneous mouse hepatomas (Emmelot and Bos, 1957*a*).

Succinate oxidation.—As shown in Table VIII the oxidation of succinate was less than that of glutamate. The P : O ratio obtained with succinate in the absence of glucose hexokinase varied from 0·8–1·4, that is 40–70 per cent of the theoretical value. The average oxygen consumption in the presence of succinate amounted to 24 μatoms O/mg. N/hour for BY 252 rat hepatoma and to 45 μatoms O/mg. N/hour for rat liver mitochondria.

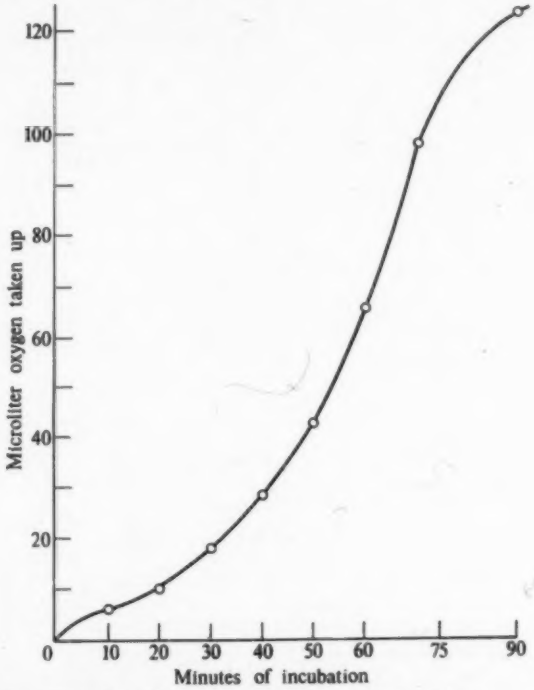

FIG. 14.—*Oxidation of hexanoate by BY 252 rat hepatoma mitochondria.*
Mitochondria isolated in 0·25 M sucrose containing 10^{-3} M EDTA; 0·66 mg. mitochondrial N present in the flasks.
Incubation as described (Emmelot, 1957*a*); hexanoate (2 μmoles), L-malate (0·0003 M), ATP (0·0007 M) and DPN (0·001 M) present in addition.
Oxygen consumption corrected for that obtained in the absence of hexanoate.

Fatty acid oxidation.—It has further been found that the BY 252 hepatoma mitochondria, isolated in 0·25 M sucrose plus 0·001 M EDTA, may be capable of oxidizing hexanoate in the presence of a catalytic amount (0·0003 M) of L-malate. A marked lag period during the first 30 minutes of incubation was observed (Fig. 14). This phenomenon suggests that during the initial period of incubation the mitochondria lack a sufficient concentration of a critical factor that has to be built up before oxidation can proceed at a maximal rate. In some of the early experiments the mitochondria from transplants, which had been growing for 10 days or even longer, were apparently unable to overcome this

lag phase, and oxygen consumption proceeded only very slowly during the whole period of incubation. The nature of the limiting factor is under further study; so far addition of ATP, coenzyme A, DPN, flavineadenine dinucleotide or nicotinamide has been without result. Octanoate dissolved in a 5 per cent serum albumin solution and added to the flasks (2 μmoles fatty acid, final concentration albumin 0·3 per cent) was also oxidized after a lag period of low O_2-uptake. In the latter case only 11 μl O_2 were consumed in the first 30 minutes, whereas 34, 50, 58 μl O_2 were taken up in each following half hour interval, so that after 4 hours a total of 440 μl O_2 was consumed after which the respiration slowed down (1·02 mg mitochondrial N/flask; 9 days' transplant). In more recent experiments carried out with mitochondria from 6–7 day-old transplants, hexanoate (no albumine) was oxidized very vigorously since, for instance, 18, 25, 34, 43, 44 and 50 μl. of oxygen were consumed during successive 10 minute intervals (total 214 μl. O_2 in 60 minutes, 0·82 mg. N). In the presence of octanoate, added as albumin complex, 210 μl. O_2 were consumed in 120 minutes (0·62 mg. N; 47, 60 and 103 μl. O_2 in the three successive 40 minute intervals) and in the presence of β-hydroxybutyrate (24 μmoles, no albumin) 240 μl. O_2 (0·73 mg. N; 45, 75 and 125 μl. in three successive 40 minute intervals) were taken up.

When oxidation proceeded at its best, 33, 22 and 23 μatoms oxygen were consumed/mg. N/hour, in the presence of hexanoate, octanoate and β-hydroxybutyrate, respectively. The function to oxidize the three fatty acids was apparently dependent upon the physiological state of the tumour transplants since the best results were obtained with small transplants which had been grown for maximally 7 days after grafting. A lag phase in the O_2 consumption of the tumour mitochondria, in contrast to normal rat and mouse liver mitochondria, was, however, always observed.

The latter oxidized palmitate (0·4 μmole), added singly or as an albumin complex, but we have as yet observed no definite oxidation of this substrate by the BY 252 hepatoma mitochondria.

Respiration of slices.—The endogenous respiration of *slices* of intraperitoneal transplants of the BY 252 rat hepatoma was similar to that of livers of the strain R-Amsterdam rat. Glucose always inhibited the respiration of the hepatoma tissue (Crabtree effect; Crabtree, 1929) but, like pyruvate, stimulated that of liver (Table IX). In a number of experiments glucose had no effect on liver respiration. Pyruvate did not affect the oxygen consumption of the hepatoma. Succinate stimulated liver respiration approximately 3-fold but had a much smaller effect in the case of the hepatoma (1·5-fold stimulation).

TABLE IX.—*Respiration by slices of the BY 252 rat hepatoma and of rat liver*

Fifteen mg., respectively 18 mg., dry weight slices incubated in 1·6 ml. Krebs-Ringer phosphate (0·02 M) buffer of pH 7·4 at 37° C. Twenty μmoles glucose, pyruvate and succinate added as indicated.

	Microatoms oxygen consumed after minutes			
	BY 252 hepatoma		*Rat liver*	
Addition	60	90	60	90
None . .	4·9	7·2	5·8	8·3
Glucose . .	3·9	6·2	7·0	10·7
Pyruvate . .	5·0	7·6	6·7	10·0
Succinate . .	7·2	11·2	16·2	24·0

Primary rat and primary and transplanted mouse hepatomas: DPN-linked oxidations.—Mitochondria prepared from primary DAB-induced rat hepatomas of the solid type also oxidized glutamate in the presence of DPN. Succinate was oxidized by these mitochondria at a similar or faster rate than glutamate. Mitochondria from subcutaneous transplants of four different mouse heptomas which originated five or more years ago in the livers of female CBA mice either spontaneously (tumours T 26473 and T 28012) or following the administration of AAT (tumours CBA 71 and CBA 80) were likewise active. For pyruvate oxidation compare below and Emmelot and Bos (1957*a*).

Fatty acid oxidation of liver and hepatoma mitochondria from rats fed DAB.—Mitochondria from livers of rats fed DAB (hepatomas removed or not yet present) oxidized octanoate regardless of the fact whether the livers showed severe or mild steatosis and/or were affected by cirrhosis or other pathological changes (Table X); it was not necessary to add DPN or a primer of fatty acid oxidation. These results indicate, first, that the fatty infiltration was not due to a diminished capacity of the neoplastic liver mitochondria to oxidize the endogenous fatty acids and, secondly, that these particles contained enough endogenous substrate and DPN to generate ATP for the initial conversion of octanoate to octanoyl-coenzyme A, and for the fatty acid oxidation *per se*. Mitochondria from the "hyperplastomas" oxidized octanoate and β-hydroxybutyrate but these particles were more or less dependent upon added DPN for full activity. The primary rat hepatoma mitochondria as a rule did not oxidize octanoate (Emmelot and Bos, 1957*a*); the oxidation that has been noted in a number of cases was, however, comparable in rate to that of normal liver mitochondria so that these positive results were not likely to be due to the presence of normal liver cells in the particular hepatoma. An uptake of oxygen in the presence of DL-β-hydroxybutyrate could be obtained regularly. In those cases in which oxidation of octanoate could not be achieved and in which the oxidation of β-hydroxybutyrate was small (approximately 3 μatoms of oxygen taken up in 60 minutes under special conditions, compare Emmelot and Bos, 1957*a*) the histological diagnosis was invariably malignant solid (adeno or mixed) hepatoma. Positive octanoate and moderate to high β-hydroxybutyrate oxidation was obtained with mitochondria which appeared to be isolated from solid trabecular hepatomas which resembled liver tissue, though some mitochondrial preparations of such tumours were not so active. In mitochondrial preparations of two of the tumours of the latter type extremely low free ATPase activity has been found.

Fatty acid oxidation of liver and hepatoma mitochondria from mice fed AAT.—The various stages of the neoplastic process have also been followed in livers of mice receiving AAT (Table XI). Before macroscopic lesions were present octanoate oxidation was diminished because endogenous citric acid cycle intermediates, which through the oxidative generation of ATP must prime fatty acid oxidation, were apparently missing. This was concluded from the fact that α-oxycaproate or L-malate had to be added as a primer in these experiments in order to obtain oxidation. The mitochondria from these livers were more labile than normal mouse liver mitochondria since washing with a KCl-phosphate buffer, instead of isotonic sucrose, destroyed the ability of the former, but not of the latter, particles to oxidize octanoate. As soon as pathological changes were visible the liver particles needed added DPN for activity (Emmelot, 1954). The requirement for DPN and primer in the octanoate oxidation was not observed

TABLE X.—*Oxidative properties of mitochondria from pre-neoplastic liver, hyperplastomas and hepatomas of rats fed DAB*

α-OC stands for α-oxycaproate, *S* for 0·25 M sucrose, *s* + *v* for 0·25 M sucrose containing 0·001 M EDTA and BHB for β-hydroxybutyrate. The substrates marked † have been incubated in the presence of 0·0003 M L-malate to ensure complete oxidation of the C_2 fragments; α-OC or L-malate were present to prime octanoate oxidation. Conditions: Emmelot and Bos (1955, 1957*a*). The oxygen-uptake in the presence of octanoate plus α-OC or L-malate (and DPN) has been corrected for that obtained in the presence of α-OC or L-malate (and DPN) alone. Time of incubation 45–60 minutes.

Mitochondria from	Isolation medium (mg. mitochondrial N/flask)	Substrate	Oxygen uptake (mm.³) DPN absent	Oxygen uptake (mm.³) DPN present
Liver, DAB 10 months, hepatoma removed (steatosis, local hyperplasia)	S (0·81)	Octanoate	57	..
		Octanoate + α-OC	84	131
Liver, DAB 10 months, no hepatoma (steatosis, local hyperplasia, cirrhosis)	S (0·97)	Octanoate	87	..
		Octanoate + α-OC	84	112
Hyperplastoma	S (1·08)	Octanoate	20	..
		Octanoate + α-OC	18	111
Corresponding liver (severe steatosis, no necrosis or cirrhosis)	S (1·53)	Octanoate	120	..
		Octanoate + α-OC	112	150
Hyperplastoma	S (1·78)	Octanoate	71	..
		Octanoate + α-OC	65	177
Corresponding liver (mild steatosis, some regeneration, glycogen normal)	S (1·38)	Octanoate	93	..
		Octanoate + α-OC	107	129
Hyperplastoma	s + v (1·28)	Octanoate†	..	121
Hyperplastoma	s + v (0·82)	DL-BHB	..	108*
Hyperplastoma mixed with solid hepatoma	s + v (1·18)	DL-BHB†	..	130
		Pyruvate†	..	127
Solid trabecular hepatoma, resembling liver	s + v (1·21)	DL-BHB	..	84
		Octanoate†	..	105
Solid (malignant) hepatoma	s + v (1·42)	DL-BHB†	6	33**
		Octanoate†	0	0**
		Pyruvate†	0	95**
Solid (malignant) hepatoma	s + v (1·08)	Pyruvate†	..	113

* 5·6 μmoles acetoacetate produced.
** Extra ATP added from side arm of respirometers (compare Emmelot and Bos, 1957*a*).

with the corresponding rat liver mitochondria which may indicate that the latter particles were more intact. The primary mouse hepatoma particles (Table XI) did not oxidize L(+)-β-hydroxybutyrate and octanoate but remained capable of oxidizing the D(−)isomer of β-hydroxybutyrate and pyruvate.

Fatty acid oxidation of spontaneous and transplanted mouse hepatoma mitochondria.—The different abilities of mitochondria isolated from these tumours to oxidize fatty acids has been described earlier (Emmelot and Bos, 1955, 1957*a*).

Oxidative phosphorylations.—The free ATPase activities of the mitochondria from the various tumours depressed the efficiency of the oxidative phosphory-

TABLE XI.—*Oxidative properties of mitochondria from pre-neoplastic, neoplastic livers and hepatomas of CBA mice fed AAT*

Abbreviations as used in Table X; (KCl-P) indicates that the second washing of the mitochondria has been carried out with KCl-phosphate buffer instead of sucrose. Liver plus adhering hepatoma has been used in the first 10 experiments.

Mitochondria from	Isolation	Amount of mitochondria (mg. N/flask)	Substrate	Oxygen uptake (mm.³) DPN absent	Oxygen uptake (mm.³) DPN present
Livers of controls (1 year old)	S	1·34	Octanoate	120	..
			Octanoate + α-OC	112	..
	S(KCl-P)	1·34	Octanoate	82	..
			Octanoate + α-OC	130	..
3 livers, AAT 8 mo ("normal")	S	1·21	Octanoate	10	..
			Octanoate + α-OC	93	..
2 livers, AAT 9 mo ("hypertrophic")	S	1·80	DL-BHB	97 (6·6)*	92
3 livers, AAF 12 mo (neoplastic foci)	S	1·38	Octanoate†	..	258
	S(KCl-P)	1·38	Octanoate†	..	16
3 livers, AAT 10 mo (neoplastic foci)	S	1·36	Octanoate†	5	109
3 livers, AAT 11 mo (one small hepatoma)	S	1·25	Choline	74	108
3 livers, AAT 11 mo (many small hepatomas)	S	1·11	Octanoate	4	13
			Octanoate + α-OC	3	106
11 livers, AAT 12 mo small and large hepatomas (2 separate experiments)	S	1·30; 1·80	D-BHB	0; 0	91; 76
			L-BHB + α-OC	0; 0	0; 0
3 livers, AAT 13 mo (large hepatomas)	S	1·51	DL-BHB	0	77 (6·9)*
7 livers, AAT 13 mo (small and large hepatomas)	S	1·80	Octanoate†	..	110 (0·5)*
3 hepatomas, with small amount of adhering liver	S	1·19	Octanoate + α-OC	16	137
3 hepatomas, dissected from 2 livers	S	1·09	Octanoate	3	8
			Octanoate + α-OC	5	15
Hepatomas from 11 livers (two separate experiments)	s + v	1·71; 1·23	DL-BHB	0; 5	83; 106 (7·2)*
			Octanoate†	5; 3	7; 4
			Pyruvate†	..; ..	105; 124
Hepatomas from 3 livers	s + v + NAA	1·03	L-Glutamate‡	13	84

* μMoles acetate produced.
† In the presence of 0·0003 M L-malate, corrected for the oxygen in the presence of malate alone.
‡ Incubated during 20 minutes.

lations when incubation was carried out in the absence of the ATPase inhibitor fluoride and the ATP-trapping system glucose-hexokinase (Table XII). It was therefore not surprising that the rate of oxidation of these particles was much less dependent upon added phosphate and ADP than in the case of normal liver mitochondria. Addition of fluoride or glucose-hexokinase markedly favoured the P : O ratios. Similar effects have been demonstrated with liver mitochondria damaged by mild ageing or treatment with electrolyte. A more satisfactory

TABLE XII.—*Oxidative phosphorylation by tumour mitochondria ; effect of hexokinase on the P : O ratio*

Mitochondria isolated in 0·25 M sucrose containing 10^{-3} M EDTA ($s + v$) or in raffinose-dextran-heparine-EDTA ($r + d$). Incubation as described (Emmelot, 1957*a*).

Glu = glutamate, Succin. = succinate, G-Hex. = glucose and hexokinase.

Mitochondria from tumour	Isolation medium	Substrate	Addition	ΔP µmoles, resp. µatoms/mg. N/20	ΔO µmoles, resp. µatoms/mg. min.	P : O
T 26473	$s + v$	Glu	..	6·3	7·0	0·9
			G-Hex.	12·6	7·0	1·8
T 28012	$s + v$	Glu	..	7·3	7·3	1·0
			G-Hex.	14·4	7·2	2·0
	$s + v$	Succin.	..	6·6	7·3	0·9
			KF(10^{-2}M)	9·4	6·1	1·5
Primary rat hepatomas (solid type)	$s + v$	Glu	..	6·3	6·3	1·0
			G-Hex.	11·6	6·4	1·8
	$r + d$	Glu	..	9·0	6·0	1·5
		Succin.	..	10·8	9·0	1·2
S_3A ascites carcinoma	$s + v$	Glu	..	7·5	5·0	1·5
			G-Hex.	11·8	5·1	2·3
UV256 sarcoma	$s + v$	Glu	..	4·2	4·2	1·0
	$r + d$	Glu	..	6·2	4·1	1·5
	$r + d$	Glu	..	10·4	4·7	2·2
		Succin.	..	12·6	8·4	1·5

P : O ratio with primary rat hepatoma and sarcoma mitochondria could also be obtained by isolating the particles in the raffinose dextran-heparine-EDTA medium, which is said to protect the structure of mitochondria (Birbeck and Reid, 1956).

Is "lability" an exclusive property of isolated tumour mitochondria ?—All the present results indicate that the integrated enzymic functions of the tumour mitochondria were less intact than those of normal liver mitochondria. Apart from the possibility to induce a similar change in isolated liver mitochondria by noxious treatment such as ageing or freeze-thawing, isolated mitochondria from pathological livers other than neoplastic, may also show an enhanced lability. Dianzani (1954, 1955), for instance, observed a loss of DPN and reduced P : O ratios with mitochondria from experimental fatty livers. We have encountered high free ATPase activity in liver mitochondria from normal Odz × dz hybrid mice (Emmelot *et al.*, 1956) ; these particles were in many cases unable to oxidize octanoate. DPN addition has been found necessary to obtain fatty acid oxidation by mitochondria isolated from the liver of two-year old O_{20} and CBA mice (Emmelot and Bos, 1955) and glutamate oxidation by liver mitochondria from apparently healthy rats (Kielley, 1957). It should be noted, however, that the enhanced lability was not inherent to the liver mitochondria of the mice (strain CBA) and rats (strain R-Amsterdam) with which the present and earlier experiments were carried out.

Guinea-pig liver mitochondria.—A marked lability has also been observed in mitochondria from livers of guinea-pigs of a partly inbred colony kept in our Institute. Sucrose mitochondria prepared from these livers showed no

detectable glucose-6-phosphatase activity and since the microsomal fraction contained a high level of the latter enzyme, it was concluded that the mitochondria were practically devoid of microsomal contamination. Marked free ATPase activities were frequently encountered in the mitochondrial preparations, and DNP stimulated the ATP-dephosphorylation of the guinea pig liver mitochondria to a smaller extent than the corresponding process of mouse and rat liver mitochondria. Glutamate was oxidized at a high rate only when DPN was added; in the absence of the coenzyme only 10–20 per cent of the oxygen uptake of the DPN-fortified particles was noted. Isolation of the particles in the presence of 0·001 M EDTA did not improve this situation, but after isolation in 0·25 M sucrose containing 0·01 M EDTA, the (higher) oxygen consumption (Table XIV) was only slightly stimulated by DPN.

The effect of thyroxine on the oxidative phosphorylation of hepatoma and guinea-pig liver mitochondria.—Thyroxine has been found to be without effect on the oxidative phosphorylation of freshly-prepared liver mitochondria from the rat (Martius and Hess, 1955; Klemperer, 1955). Only after a pretreatment of the particles, such as a preliminary incubation with thyroxine at 0° C or suspension in a hypotonic medium (Tapley, Cooper and Lehninger, 1955) does the hormone uncouple the oxidations from the accompanying phosphorylations in the respiratory chain. This has been taken to mean that some time is needed for thyroxine to penetrate into the mitochondria via the intact membranes of fresh liver particles. The oxidative phosphorylations of fresh hamster liver mitochondria, washed once with electrolyte instead of sucrose, have been found by Hoch and Lipmann (1954) to be readily uncoupled by thyroxine without further pretreatment and these particles were, therefore, considered to be "leaky". However, the procedures of pretreatment used to bring about the uncoupling effect are not only noxious to the mitochondrial structure but also to its integrated enzymic performance, which is closely related to structure. The observation that the thyroxine-sensitive hamster liver mitochondria readily loose DPN and cytochrome *c* whereas the insensitive rat liver mitochondria do not, fits in not only with the "leakiness" but also with a reduced overall functional intactness of the particles. Whatever the explanation may be, it is evident that damaged mitochondria (compare Table XIII, 1st experiment) do respond readily in terms of uncoupling of their oxidative phosphorylation to thyroxine. It would therefore be of interest to study the effect of thyroxine on the oxidative phosphorylations of these hepatoma mitochondria which give evidence of an increased lability as compared with fresh liver mitochondria. Previous experiments have shown that thyroxine may indeed uncouple the oxidative phosphorylations of fresh untreated tumour mitochondria (Emmelot and Brombacher, 1957). In the present experiments the phosphorylations accompanying the oxidation of glutamate by fresh liver mitochondria from our rats were sometimes slightly depressed by thyroxine ($7{\cdot}5 \times 10^{-5}$ M), *i.e.* 25 per cent at its most, whereas a drop of 40–60 per cent in the P : O ratio of the BY 252 rat hepatoma mitochondria was consistently noted. The oxidative phosphorylations of fresh mouse liver mitochondria were not affected by thyroxine. Neither did thyroxine affect the P : O ratios obtained with mitochondria from the transplanted mouse hepatoma CBA 71. The phosphorylations accompanying the oxidation of β-hydroxybutyrate of T 28012 mitochondria were not affected, but with glutamate as substrate uncoupling in the presence of thyroxine was noted. The hormone markedly depressed the oxidative phosphorylation of T 26473

TABLE XIII.—*Effect of thyroxine on the oxidative phosphorylation of hepatoma mitochondria*

Mitochondria isolated in 0·25 M sucrose containing 10^{-3} M EDTA. 0·5–0·9 mg. mitochondrial N present in the various experiments.
In all experiments freshly isolated mitochondria were used, except in one case in which liver mitochondria were sonicated during 5 minutes prior to incubation. Glutamate (GLU) oxidation in histidine buffer, as described previously (Emmelot, 1957*a*). β-Hydroxybutyrate (BHB) and pyruvate oxidation in phosphate buffer (Emmelot and Brombacher, 1957). DPN added in a final concentration of 10^{-3} M. Thyroxine ($7·5 \times 10^{-5}$ M) present in the main compartment except in two experiments marked *, in which the hormone was added from the side arm of the respirometer flasks after the time allowed for temperature equilibration. KF (10^{-2} M), EDTA (10^{-3} M) and glucose-hexokinase were added as indicated. Incubation during 15–20 minutes at 27° C; pH 7·4.

Mitochondria from	Substrate	Addition	Thyroxine	ΔP (μmoles)	ΔO (μatoms)	P : O
Rat liver	GLU	..	−	14·2	6·7	2·1
			+	11·1	6·5	1·7
		(Sonicated mitochondria)	−	13·4	7·8	1·7
			+	1·5	6·4	0·2
BY 252—rat hepatoma	GLU	..	−	6·9	4·3	1·6
			+	3·0	3·2	0·9
	GLU	..	−	8·7	5·8	1·5
			+	1·7	2·8	0·6
		10^{-3} M EDTA	−	10·5	7·0	1·5
			+	5·1	4·1	1·2
CBA71 — mouse hepatoma	Pyruvate (L-malate)	Fluoride, G-Hex.	−	10·8	5·4	2·0
			+	9·8	5·4	1·8
	GLU	Fluoride	−	4·8	3·6	1·3
			+	3·6	2·6	1·4
T 26473 — mouse hepatoma	BHB	Fluoride, G-Hex.	−	5·4	2·0	2·7
			+*	3·0	2·5	1·2
	BHB	Fluoride, G-Hex.	−	5·1	2·4	2·1
			+*	1·5	2·0	0·75
	BHB	Fluoride, G-Hex.	−	6·0	2·5	2·4
			+	1·9	2·1	0·9
	GLU	Fluoride	−	5·8	4·3	1·3
			+	0·7	2·2	0·3
T 28012 — mouse hepatoma	BHB	Fluoride, G-Hex.	−	7·7	3·7	2·1
			+	7·2	3·6	2·0
	GLU	G-Hex.	−	6·8	4·5	1·5
			+	2·7	4·9	0·5
	GLU	Fluoride	−	3·2	3·2	1·0
			+	0	1·6	0·0

mitochondria. Thyroxine was present in the main compartment of the respirometers in all except two experiments with T 26473 mitochondria in which the hormone was added from the sidearm together with the oxidizable substrate after temperature equilibration had been reached. This procedure was followed in order to reduce the contact between the particles and the hormone to a minimum. Since uncoupling still occurred under the latter conditions, it may be concluded that thyroxine acted momentarily. The reasons for the difference in effect of thyroxine on the various hepatoma mitochondria is not clear, there is some evidence that it may be connected with differences in the intrinsic lability of the particles.

It has been shown in an earlier paper (Emmelot and Bos, 1958) that thyroxine inhibited the oxidation of glutamate by mouse liver mitochondria but that in the presence of 0·001 M DPN hardly, if any, inhibition resulted. By contrast, thyroxine inhibited the glutamate oxidation of the tumour particles markedly in most of the present experiments in spite of the excess DPN being added. The latter phenomenon might be related to the weak binding between DPN and the hepatoma particles.

A marked inhibition of glutamate oxidation in the presence of thyroxine and DPN has also been noted with the guinea pig liver mitochondria, which, like the hepatoma and unlike the mouse and rat liver mitochondria, need DPN for optimal oxidation in the absence of thyroxine. The resemblance between fresh guinea pig liver and hepatoma mitochondria was the more striking since the oxidative phosphorylations of the former particles were also completely abolished by thyroxine (Table XIV). EDTA counteracted (compare Park *et al.*, 1956) the uncoupling of the oxidative phosphorylations of the guinea pig liver mitochondria (Table XIV), the BY 252 rat hepatoma mitochondria (Table XIII) and hypotonically-treated thyroxine-sensitive mouse liver mitochondria (Table XIV).

In summarizing the results of the experiments with thyroxine it may be stated that they lend additional support to the view that mouse and rat hepatoma mitochondria may be functionally less intact than the corresponding liver mitochondria, but that this property is not specific for the tumour mitochondria.

DISCUSSION

Since in the present investigation mainly " solid " tumours (solid in the sense of a coherent mass of cells in contrast to the ascites form in which a tumour may be grown) have been used, it seems well to consider briefly the statement of Warburg (1956) that only ascites tumours should be studied and that " solid " tumours—and especially " solid " spontaneous tumours—need no longer be subjected to metabolic examinations to-day since the " solid " tumours are usually so impure histologically in that normal cells are present. Considered in regard to transplanted " solid " tumours, this statement seems to us to be incorrect for the following reasons. Host fibrous tissue (stroma) is present in all organoid formations whether tumour or not. However, in rapidly growing transplanted carcinomas the fibrous elements are less developed than in the slower growing primary tumour but not necessarily more than in the parent normal tissue. Continuous transplantation can be considered as constituting a means of obtaining the medullary—as opposed to the scirrhus—type of the " solid " carcinoma. Thus, as long as normal tissues, not grown in tissue culture, are used for metabolic studies, the use of transplanted

TABLE XIV.—*Effect of thyroxine on the oxidative phosphorylation of fresh guinea-pig liver and hypotonically-treated mouse liver mitochondria in the absence and presence of EDTA*

Glutamate oxidation in histidine buffer in the presence of DPN (10^{-3} M) as described previously (Emmelot, 1957*a*). 0·005 M and 0·0025 M $MgCl_2$ were added, respectively, in the experiments with the guinea-pig liver and the hypotonically-treated mouse liver mitochondria. The latter were obtained by suspending fresh mouse liver mitochondria in 0·075 M sucrose during 10 minutes at 0° C prior to use. EDTA added to the respirometers or included in the isolation medium as indicated. 0·3 Ml. of the mitochondrial suspensions corresponding to 250–300 mg. of wet weight liver were added to give a final volume of 1·6 ml. S stands for 0·25 M sucrose.

Mitochondria from	Isolation	Addition	Thyroxine	ΔP (μmoles)	ΔO (μatoms)	P : O
Guinea-pig liver	S	..	—	10·2	6·7	1·5
			+	2·0	4·3	0·5
		G-Hex.	—	12·3	5·8	2·1
			+	2·4	4·5	0·5
Guinea-pig liver	S	..	—	9·8	6·1	1·6
			+	0·0	3·8	0·0
	S + 10^{-3} M EDTA	..	—	12·6	5·9	2·1
			+	1·5	3·7	0·4
	S + 10^{-2} M EDTA	..	—	9·9	5·5	1·8
			+	8·0	5·0	1·6
	S + 10^{-2} M EDTA	(No DPN)	—	10·2	5·1	2·0
			+	5·0	2·5	2·0
Guinea-pig liver	S	..	—	12·8	8·0	1·6
			+	0·0	4·9	0·0
		10^{-3} M EDTA	—	14·6	7·3	2·0
			+	8·8	5·5	1·6
		10^{-2} M EDTA	—	13·8	6·0	2·3
			+	9·8	4·2	2·3
Mouse liver hypotonically treated	S	..	—	11·7	9·8	1·2
			+	1·4	7·0	0·2
		10^{-3} M EDTA	—	13·3	7·7	1·7
			+	10·0	6·2	1·6

medullary tumours is fully warranted if the latter are free from normal cells besides the stroma. Metabolic investigations on normal cells cultured *in vitro* should be considered in the light of the accumulating evidence that enzyme functions, present in the intact tissue, may be lost (Perske, Parks, and Walker, 1957). Now, although early transplant generations of solid tumours may occasionally contain some rests of normal tissue, most well-established transplanted tumours are free from the latter, as judged by all available histological criteria. We can safely state that this is the case with the BY 252 rat hepatoma and other transplanted tumours used in our investigations.

The fact remains, however, that the cell population of a transplanted tumour, in contrast to that of the ascites tumour, may not be uniform in that differences in chromosome number, metabolic activities and morphology between individual cells may exist to some extent (references in Klein, 1956, and Sylvén and Malmgren, 1957). These differences between the tumour cells may be due to their deoxyribonucleic acid-content (compare Kit and Griffin, 1958) but they may, partly at least, also be caused by mechanical factors which govern the supply of nutrients and oxygen and, subsequently, the synthesis of enzymic-active proteins. However, all these cells are *tumour* cells and the study of their (average) metabolic activities is justified since there is no evidence whatsoever to suggest that a more uniform cell population selected from the "solid" tumour by environmental pressure, as seems to be the case in ascites cell formation, represents the type of tumour cell as it originated in first instance. On the contrary, since most tumours are not convertible to the ascites form, one may assume that the particular requirements for this conversion are not met with in large cell populations which are definitely tumourous. Only the most anaplastic tumours are easily convertible, whereas others need several passages before complete transformation into the ascites form results (Klein, 1956). Physiological adaptation and mutational adjustments seem to be involved in the transformation. Moreover, ascites tumour cells, as well as *in vitro* cultured free cells, have lost their original relation to the host stroma, a situation which makes these cells *ipso facto* different from the original cells of the coherent tumour. The absence of such an organized architecture may result in a profound alteration of certain metabolic properties as, for instance, the active transport of metabolites into the cells. The study of ascites cells thus furnishes information about a very particular, but not about *the* cancer cell. In fact, to seek information about the metabolism of *the* tumour cell may be an illusory ideal, as demonstrated, for instance, by the varied responses of different tumours in the face of a chemotherapeutical challenge and by the increasing evidence of differences in the metabolic activities between various tumours (compare for ascites tumours: Forssberg and Révész, 1957; Purdom, Ambrose and Klein, 1958). Such differences among tumours may give information about the grade of malignancy, as exemplified biologically by the degree of differentiation, growth rate, potency to metastasize and biochemically by the loss of certain enzymic functions and the acquirement to capture and use preformed metabolites of the host to the advantage of metabolic channelling of precursors in alternative directions, cellular adhesiveness in relation to membrane charge (Purdom *et al.*, 1958), etc.

The ascites tumour furnishes a powerful tool for investigation of many highly interesting aspects of the cancer problem, but by its very nature the ascites tumour should not monopolize biochemical cancer research.

From the observations collected in this paper, which are mainly concerned with those biochemical properties dependent upon the integrated structural and enzymic functions of mitochondria, a number of conclusions can be drawn.

(i) Since our data concerning the mitochondrial glutamic dehydrogenase of the BY 252 rat hepatoma are in marked contrast to those published on the Novikoff hepatoma (Allard *et al.*, 1957), one arrives at the situation that two transplanted undifferentiated tumours of the same type and species differ qualitatively in an important enzyme. This represents an exception to the reverse of the principle of metabolic uniformity among undifferentiated tumours.

(ii) Sufficient evidence on differences in enzymatic activities between isolated mitochondria of various hepatomas is now available to conclude that no general statements about possible divergencies in mitochondrial activities can be derived from a comparative study of normal liver and one particular hepatoma unless such a conclusion is checked on several tumours of the same type. The same consideration may also apply to the metabolic activities of cellular structures other than mitochondria. It has been shown, for instance, that the microsomes in a 15,000 × g supernatant of the mouse hepatoma T 28102 are moderately active in incorporating radioactive leucine whereas those of T 26473 and of primary rat hepatomas were only very slightly active in this respect (Emmelot, 1957*b*). Very recently we have found a high incorporation of amino acid, resembling that obtained with liver preparations, in microsomes of a 15,000 × g supernatant of young, but not of old, transplants of the BY 252 rat hepatoma. It is interesting that the capability of the mitochondria from these tumours to oxidize fatty acids exactly parallels the amino acid incorporation studies, which suggests that the "lability" is common to both mitochondria and microsomes.

(iii) It has been shown that mouse and rat hepatoma mitochondria, as a class, tend to show an increased lability in comparison with the corresponding liver mitochondria. The lability of the particles manifests itself by a more or less ready loss of DPN from the particles (compare also Wenner and Weinhouse, 1953; Kielley, 1952), increased free ATPase activity in the presence of Mg^{2+}, decreased P : O ratios in the absence of fluoride and glucose-hexokinase, more exacting conditions for obtaining fatty acid oxidation (compare also Emmelot and Bos, 1955) and an abolishment of the oxidative phosphorylations of most of the fresh tumour particles in the presence of thyroxine. It should be emphasized that in several of these respects quantitative differences in the degree of variance from "normal" may be encountered among the mitochondria from various hepatomas (Emmelot and Bos, 1955; 1957*a*, *b*, *c*) and even among mitochondrial preparations from different transplants of the same hepatoma. As regards the latter aspect, it is suggested that necrobiotic phenomena might be involved in the case of the BY252 hepatoma.

Various observations suggest that the enhanced lability of the hepatoma mitochondria manifests itself *in vitro*. This applies, for example, to the loss of DPN from the BY 252 hepatoma particles. Moreover, direct analysis, carried out in the presence of NAA to prevent the enzymic destruction of DPN by DPNase, has shown that mitochondria from the primary hepatomas of our rats may contain from 25–50 per cent less DPN than normal rat liver mitochondria (compare also Glock and McLean, 1957) but that sometimes even no trace of DPN was present (assay with alcohol dehydrogenase). Since ascites tumour cells and tumour slices, including those of hepatomas, do oxidize fatty acids, we assume that the loss of fatty acid oxidation observed with certain mitochondrial preparations is not due to the absence of the necessary enzymes but to the damage inflicted upon the integrated function of the particles *in vitro*. Moreover, it has been shown that tumour mitochondria isolated in 0·25 M sucrose, unlike liver particles, do not oxidize octanoate, but that after isolation in 0·25 M sucrose containing 0·001M EDTA vigorous oxidation ensues (Emmelot and Bos, 1955). The experiments on the fatty acid oxidation of the BY 252 rat hepatoma mitochondria are another point in case. It should be noted that the reported absence of glutamic dehydrogenase activity from the Novikoff hepatoma mitochondria is

not likely to be due to the lability of these particles. Disruption of the particles would bring this enzyme, which is rather stable, in solution; Allard *et al.* (1957) were, however, unable to demonstrate enzyme activity in homogenates of the Novikoff hepatoma. What has been said about the hepatoma mitochondria also applies to other tumour mitochondria (e.g. Emmelot and Brombacher, 1956) but it should be emphasized that lability is not an exclusive property of isolated mitochondria from neoplastic tissues.

(iv) Apart from a quantitative or qualitative difference in enzyme function as a result of the greater lability of the isolated hepatoma mitochondria, such particles may also possess a smaller content of certain enzymes than liver mitochondria. This applies, for example, to the Mg^{2+}-activated ATPase of the sonic disrupted and to the DNP-activated ATPase of intact liver and tumour mitochondria. The possibility may, as yet, not be excluded that an inhibitor of the ATPase is present in the tumour mitochondrial preparations. In the former case disorganization of the structure has been induced deliberately in order to uncover the potential content of the enzyme. In a homogenate of the Novikoff hepatoma the succinoxidase activity is reduced to 13 per cent of the liver level (Novikoff, 1957). Since Allard *et al.* (1957) found that the number of mitochondria present in the latter tumour amounted to 25 per cent of that present in liver, one may conclude that the average Novikoff mitochondrion contains one-half of the succinic oxidase activity of a liver mitochondrion. Our data on the BY 252 rat hepatoma and those of Schneider and Hogeboom (1950) on a transplanted mouse hepatoma are in accordance herewith. Since the succinic oxidase does not appear to be easily inactivated by *in vitro* conditions, the above observation may be interpreted as reflecting the actual mitochondrial content of the enzyme. Since succinate oxidation is less dependent upon ATP turnover than the oxidation of other citric acid-cycle intermediates, the oxygen consumption of tumour slices in the presence of succinate may give a rough measure of the succinoxidase activity of intact cells. The much greater stimulation of the oxidation of liver slices by succinate over the no substrate level as compared with that of primary (Olson, 1951) and of transplanted (Table IX) rat hepatomas indicates that a marked difference in succinoxidase content of the normal and neoplastic tissues does exist. This is due to differences in the amount of mitochondria present and in the succinoxidase content per mitochondrion. It should, however, be pointed out that the lower succinoxidase activity of the hepatomas does apparently not function as a bottle-neck in the endogenous (fatty acid oxidation; Medes, Paden and Weinhouse, 1957) respiration nor in the oxidation of carbohydrates, probably because other reactions (e.g., phosphorylation) are rate-limiting under these conditions.

(v) It has been shown that isolated mitochondria from "hyperplastomas", solid trabecular hepatomas resembling liver tissue and malignant hepatomas (solid and adeno) of the rat show a decreased functional intactness in the order given. It is known that a close correspondence between mitochondrial morphology and function exists in that structural disorganization leads to impaired function. Of the mitochondrial enzymes the glutamic dehydrogenase is located in the soluble part (matrix) of the particles whereas most Krebs-cycle and phospho-transferring enzymes are located in the insoluble part (membranes). Our primary rat hepatomas are being studied by Professor van Rijssel and Dr. de Man at Leiden University with the electron microscope (de Man *et al.*, 1959). It has been found that the

solid hepatoma mitochondria had a very heterogeneous appearance both in dimensions and fine-structure. In general the hepatoma mitochondria were smaller and present in lesser amount than liver mitochondria. Sometimes no internal membranes were seen but in other cases more "cristae" were present than in liver mitochondria. Particles of an obviously normal type and giant types were also encountered. Liver mitochondria contain an evenly dense matrix but in the hepatoma mitochondria sometimes very little matrix was present which was unevenly distributed thus giving rise to optically-empty cavities of an irregular and faded shape. It has been shown that enlarged and apparently degenerative mitochondrial structures next to small, more or less intact ones, are also present in the Novikoff hepatoma and other tumours (Howatson and Ham, 1955, 1957; Novikoff, 1957; Bernhard, 1957, 1958).

Considering the close correlation between mitochondrial structure and enzymic function, it is not difficult to envisage, first, that the enzyme content of liver and hepatoma mitochondria may vary in certain respects and, secondly, that the loss or inactivation of certain mitochondrial enzymes and co-factors *in vitro* may be due to the labile nature of at least part of the mitochondrial population of the hepatoma cells, as is suggested by the degenerative features in the fine-structure of these particles. These considerations may possibly also apply to other tumours.

What effect the observed change in fine-structure may have upon the activity of the tumour mitochondria *in situ* is not known. In so far as the mitochondrial oxidative phosphorylations are concerned no serious defects seem to result since the available evidence strongly suggests that the latter processes do occur in intact tumour cells. Addition of the uncoupling agent DNP to tumour slices or ascites cells leads to a marked rise in the oxygen consumption, an inhibition of certain metabolic reactions and to the abolishment of the Pasteur effect (Emmelot and van Vals, 1957*b*; Emmelot and Bos, unpublished).

SUMMARY

1. A transplanted rat hepatoma of the solid (hepatocellular) type—the BY 252 hepatoma—has been established. Mitochondria were isolated from this tumour and their properties compared with those of normal liver mitochondria.

The ATPase activity of the fresh hepatoma particles was not latent; DNP activated the ATPase in the presence of Mg^{2+} at 27° C but at 37° C hardly, if any, effect was noted; the ATPase of the tumour particles was already more or less completely active at the latter temperature in the presence of Mg^{2+} alone. By contrast, most of the ATPase complement of normal liver mitochondria from rats of the same strain as that in which the hepatoma had arisen, was still in a latent state at 37° C. The total ATPase content, as manifested in fresh mitochondria by Mg^{2+} plus DNP or in disrupted mitochondria by Mg^{2+} alone, was smaller in the case of the hepatoma than of the liver. DNP abolished the Pasteur effect in slices of BY 252 hepatoma and other tumours (primary rat hepatomas, a mammary ascites carcinoma and a sarcoma of the mouse). DNP also activated the ATPases of the mitochondria isolated from the latter tumours.

2. The endogenous respiration of slices of the BY 252 hepatoma was not different from that of normal rat liver. Glucose inhibited the respiration of the hepatoma (Crabtree effect). Succinate stimulated the respiration of the hepatoma for about 50 per cent but that of liver for about 300 per cent.

Oxidation and phosphorylation by the BY 252 hepatoma mitochondria were also studied. The succinoxidase activity was about half of that shown by the same amount of normal rat liver particles. The hepatoma particles possessed a considerable glutamic dehydrogenase activity, in contrast to what is reported about the Novikoff rat hepatoma. The BY 252 hepatoma mitochondria released part of their DPN-complement during incubation. Oxidative phosphorylation in the absence of glucose-hexokinase was below the normal level but could be raised to the normal level by adding the ATP-trapping system. Thyroxine exerted a marked uncoupling effect on the oxidative phosphorylation of the fresh hepatoma in contrast to that of the liver mitochondria. EDTA counteracted the uncoupling by thyroxine of the oxidative phosphorylation of the hepatoma mitochondria. The conditions necessary for obtaining fatty acid oxidation, apart from being dependent upon the age of the transplants from which the mitochondria were prepared, were more exacting in the case of the hepatoma than of the liver mitochondria.

3. From the spontaneous activation of the ATPases, the release of DPN, the lowered P : O ratios, the effect of thyroxine on oxidative phosphorylation and the conditions necessary for fatty acid oxidation, it was concluded that the isolated BY 252 rat hepatoma mitochondria were more labile *in vitro* than rat liver mitochondria.

4. Similar studies made with mitochondria from 3 transplanted mouse hepatoma strains and many primary rat and mouse hepatomas induced by DAB and AAT, respectively, revealed also the labile character of these hepatoma mitochondria. In regard to certain enzymic properties some hepatoma particles were less labile than others. However, the labile character was not an exclusive property of isolated mitochondria from neoplastic tissues since liver mitochondria from a partly inbred guinea pig strain gave also evidence of an increased lability in comparison to mouse and rat liver mitochondria.

5. A parallel biological and biochemical study of livers from rats fed with DAB was made. Mitochondria from pre-neoplastic livers behaved like normal liver mitochondria except that the free ATPase was sometimes (somewhat) enhanced and that DNP had a smaller effect in those cases in which the pathological changes were very pronounced. Non-malignant local hyperplasias (" hyperplastomas ") solid trabecular hepatomas and less differentiated solid hepatomas, next to adeno and mixed solid-adeno hepatomas were enccuntered. Of the mitochondria isolated from these types of growth, those of the former two bore more resemblance to normal liver mitochondria than the latter. The mitochondria of pre-neoplastic mouse livers gave evidence of a greater lability than those of pre-neoplastic rat liver mitochondria.

6. The implications of the present findings are discussed. It is concluded that—compared with normal rat liver mitochondria—the decreased function of the isolated hepatoma mitochondria manifests itself as a result of the *in vitro* techniques and that the labile character, as well as the diminished content of certain enzymes, may be correlated with the particular fine-structural organization of the latter particles *in situ*, as suggested by electron microscopical examination.

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REFERENCES

ALLARD, C., DE LAMIRANDE, G. AND CANTERO, A.—(1957) *Cancer Res.*, **17**, 862.
BARKER, S. B. AND SUMMERSON, W. H. (1941) *J. biol. Chem.*, **138**, 535.
BERNHARD, W.—(1957) *Klin. Wschr.*, **35**, 251.—(1958) *Cancer Res.*, **18**, 491.
BIRBECK, M. S. C. AND REID, E.—(1956) *J. biochem. biophys. Cytol.*, **2**, 609.
CLERICI, E. AND CUDKOWICZ, G.—(1956) *J. nat. Cancer Inst.*, **16**, 1459.
CRABTREE, H. C.—(1929) *Biochem. J.*, **23**, 536.
DE MAN, J. C. H., BRAAMS, W. G., WEILAND, H. TH. and EMMELOT, P.—(1959) *Ned. Tijdschr. Geneesk.* **103**, 108.
DIANZANI, D. U.—(1954) *Biochim. biophys. Acta*, **14**, 514.—(1955) *Ibid.*, **17**, 391.
EMMELOT, P.—(1954) *Jaarb. Kankeronderzoek en bestrijding Ned.*, **4**, 75.—(1957*a*) *Biochem. biophys. Acta*, **23**, 668.—(1957*b*) *Exp. Cell Res.*, **13**, 601.
Idem AND BOS, C. J.—(1955) *Rec. Trav. chim. Pays-Bas*, **74**, 1343.—(1957*a*) *Enzymologia*, **18**, 179.—(1957*b*) *Exp. Cell. Res.*, **12**, 191.—(1957*c*) *Brit. J. Cancer*, **11**, 148.—(1958) *Exp. Cell Res.*, **14**, 132.
Idem, BOS, C. J. AND BROMBACHER, P. J.—(1956) *Brit. J. Cancer*, **10**, 188.
Idem AND BROMBACHER, P. J.—(1956) *Biochim. biophys. Acta* **22**, 487.—(1957) *Ibid.*, **23**, 435.
Idem AND VAN VALS, G. H.—(1957*a*) *Z. Krebsforsch.*, **62**, 116.—(1957*b*) *Ibid.*, **62**, 63.
ERNSTER, L.—(1956) *Exp. Cell Res.*, **10**, 704, 721.
FISKE, C. H. AND SUBBAROW, Y.—(1929) *J. biol. Chem.*, **81**, 629.
FORSSBERG, A. AND RÉVÉSZ, L.—(1957) *Biochim. biophys. Acta*, **25**, 165.
GLOCK, G. E. AND MCLEAN, P.—(1957) *Biochem. J.*, **65**, 413.
GREENSTEIN, J. P.—(1953) "Biochemistry of Cancer", New York (Academic Press Inc.) 2nd edn.—(1956) *Cancer Res.*, **16**, 641.
HOCH, F. L. AND LIPMANN, F.—(1954) *Proc. nat. Acad. Sci. Wash.*, **40**, 909.
HOGEBOOM, G. H. AND SCHNEIDER, W. C.—(1953) *J. biol. Chem.*, **204**, 333.
HOWATSON, A. F. AND HAM, A. W.—(1955) *Cancer Res.*, **15**, 62.—(1957) *Canadian Cancer Conference*, **2**, 17.
HUNTER, JR., F. E.—(1956) *Int. Congr. Biochem., Brussels*, 1955, **3**, 298.
JOHNSON, M. S.—(1941) *Science*, **94**, 200.
KIELLEY, R. K.—(1952) *Cancer Res.*, **12**, 124.—(1957) *J. biol. Chem.*, **227**, 91.
KIELLEY, W. W. AND KIELLEY, R. K.—(1951) *Ibid.*, **191**, 485.
KIT, S. AND GRIFFIN, A. C.—(1958) *Cancer Res.*, **18**, 621.
KLEIN, G.—(1956) *Ann. N.Y. Acad. Sci.*, **63**, 640.
KLEMPERER, H. G.—(1955) *Biochem. J.*, **60**, 122.
LYNEN, F.—(1941) *Liebigs Ann.*, **564**, 120.—(1958) "Neuere Ergebnisse Chemie u. Stoffwechsel Kohlenhydrate", Berlin (Springer Verlag) p. 155.
MCBURNEY, R. P., WOOLNER, L. B. AND WOLLAEGER, E. E.—(1950) *Proc. Mayo Clin.*, **25**, 606.
MARTIUS, C. AND HESS, B.—(1955) *Biochem. Z.*, **191**, 326.
MEDES, G., PADEN, G. AND WEINHOUSE, S.—(1957) *Cancer Res.*, **17**, 127.
NOVIKOFF, A. B.—(1957) *Ibid.*, **17**, 1010.
OLSON, R. E.—(1951) *Ibid.*, **11**, 571.
PARK, J. H., MERIWETHER, B. P., PARK, C. R., MUDD, S. M. AND LIPMANN, F.—(1956) *Biochim. biophys. Acta.* **22**, 403.
PERSKE, W. F., PARKS, JR., R. E. AND WALKER, D. L.—(1957) *Science* **125**, 1290.
POTTER, V. R., SIEKEVITZ, P. AND SIMONSON, H. C.—(1953) *J. biol. Chem.*, **205**, 893.
PURDOM, L., AMBROSE, E. J. AND KLEIN, G.—(1958) *Nature, Lond.*, **181**, 1586.
RACKER, E.—(1956) *Ann. N.Y. Acad. Sci.*, **63**, 1017.
REID, E. AND O'NEIL, M. A.—(1956) *Brit. J. Cancer*, **10**, 287.
REYNAFARJE, B. AND POTTER, V. R.—(1957) *Cancer Res.*, **17**, 1112.
SCHNEIDER, W. C. AND HOGEBOOM, G. H.—(1950) *J. nat. Cancer Inst.*, **10**, 969.

SIEKEVITZ, P., LÖW, H., ERNSTER, L. AND LINDBERG, O.—(1958) *Biochim. biophys. Acta*, **29**, 378.
SLATER, E. C. AND CLELAND, K. W.—(1953) *Biochem. J.*, **55**, 566.
SYLVÉN, B. AND MALMGREN, H.—(1957) *Acta radiol., Stockh.*, Suppl. 124.
TAPLEY, D. F., COOPER, C. AND LEHNINGER, A. L.—(1955) *Biochim. biophys. Acta*, **18**, 597.
WARBURG, O.—(1956) *Science*, **123**, 309.
WEBER, G. AND CANTERO, A.—(1957) *Cancer Res.* **17**, 995.
WENNER, C. E. AND WEINHOUSE, S.—(1953) *Ibid.*, **13**, 21.

ADDENDUM

Since this paper went to press it has been reported by M. Birns, E. Essner and A. B. Novikoff (1959, *Proc. Amer. Ass. Cancer Res.*, **3**, 7) that, contrary to the finding of Allard *et al.* (1957) the glutamic acid dehydrogenase was present in the Novikoff rat hepatoma kept in the laboratory of the former authors. A study of the Dunning transplantable rat hepatoma LC 18/81A (Pitot, N. C., Fohn, C. H., Clark, Jr., W. H. and Farber, E., 1959, *Proc. Amer. Ass. Cancer Res.*, **3**, 52) has led to the same general conclusion as that reached in the present paper, namely "that a number of different hepatomas must be studied before it can be assumed that any particular set of biochemical or morphological peculiarities observed in one hepatoma is characteristic and distinctive for hepatic neoplasms as a group." In this connection it might be of interest to investigate whether the decrease in or the absence of certain enzymic activities are real or due to endogenous inhibitors.